NUST JOURNAL OF NATURAL SCIENCES

Volume 3 **June - Dec, 2015** **Issue: 1**

Contents

Hematological Studies in Chickens Infected with Hydro Pericadium Syndrome

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ABSTRACT

The present research project was designed to determine the hematological values in infected broiler flocks with hydropericardium syndrome from January to June, 2012 in order to adjoining areas of Hyderabad Sindh. A total of 72 containing population 96,500 broiler chickens were surveyed to observe for hematological variation during the study period. Only 30 (41.66%) flocks were found affected with Hydro pericardium syndrome. Out of these flocks, the incidence of syndrome was recorded as 5.4% during the month of January, 7.41% in February, 10.8% in March, 13% in April, 15.1% in May and 12.8% during the month of June. The hematological studies were conducted in infected broilers with hydropericardium syndrome and found increase in white blood cells (WBC) from 14.500 ± 2.298 to 25.820 ± 844.00 and concentration of erythrocytic sedimentation rate (ESR) from 2.340 ± 0.89 to 3.458 ± 0.291. Amongst Leukocytic count, showed marked increase with neutrophils and relative decrease of lymphocytes from 69.360 ± 0.152 to 55.480 ± 2.132, monocytes 4..660 ± 0.598 to 3.340 ± 270, eosinophils 1.496 ± 0.204 to 1.004 ± 0.097 and basophils 0.878 ± 0.55 to 0.734 ± 0.067 in infected birds.

Key Words: Hydro pericardium syndrome, Hematology, Broiler, Pericardial sac.

1. INTRODUCTION

The occurrence of hydropericardium syndrome (HPS) causes heavy economic losses to poultry industry in many parts of the world including Pakistan. The Hydro pericardium or Hepatitis Syndrome (HHS) was first recognized in broiler farms in Angara Goth (Goth means small town) near Karachi metropolitan city of Pakistan, in late 1987 (Jaffery et al., 1986, Jaffery et al., 1988, Anjum et al., 1989, Balamurugan and Kataria, 2004). Because the disease emerged in this specific geographic area, Hydro pericardium was initially referred to as "Angara Disease". The syndrome was spread in the densely populated broiler growing areas all over the country within six months. The outbreaks of Hydro pericardium were also recorded in Mexico in 1989 in the high density poultry producing states (Borrego and Soto, 1995, Khan et al., 2005, Khan et al., 2012).

It is an acute disease of broiler chickens of 3-5 weeks of age and caused by a group of several viruses which belong to adnenoviruses in origin. The disease is characterized Sero-jelatinous fluids in the pericardial sac (straw colored fluid). The infection results in great economic losses and high mortality rate up to 70% in all age groups of broiler. Characteristically the affected birds show gasping, depression, inability to move, off feed, paleness of wattles and combs alongwith swollen, restlessness, resting of beak pints on the ground and immediate death associated with stress (Irfan et al., 1988, Kumar et al., 2003, Peters et al., 2011). The most prominent features of the disease are dilation of pericardial sac with heavy dropsical fluid and atrophy of the myocardium. Realizing the importance and heavy economic losses due to hydro-pericardium syndrome among poultry industry, the present study was carried out to record the hematological variation and clinical changes for the confirmation of the disease in naturally infected broiler chickens (Jaffery et al., 1988, Anjum et al., 1989, Rani et al., 2011, Tehrani et al., 2012).

2. AIMS AND OBJECTIVES

(a) To evaluate the hematological variation of hydro pericardium syndrome infected broilers flocks.

(b) To record the clinical findings of hydro pericardium syndrome in infected broilers.

(c) To investigate the heart (pericardial sac) condition during infection.

(d) To observe the gross pathological lesions in different visceral organs during autopsy.

3. MATERIALS AND METHODS

The present survey was conducted to locate the pockets of hydro pericardium syndrome in broiler flocks during January to June 2012. The valuable information was under taken for the purpose total of 72 broiler farms out of these 30 farms were found infected with hydro pericardium were visited with the interval of 10 days for blood samples randomly collected from ailing birds for the confirmation of hydro pericardium mostly 3-6 weeks of age in the flocks ranging from 1000-3000 at each broiler farms. The broiler flocks which were naturally affected with hydro pericardium were selected for

hematological parameters including (i) Total count (ii) Total leukocytic count (iii) Differential leukocytic count (iv) Haemoglobin concentration (Hb) (v) Erythrocytic sedimentation rate (ESR) (vi) Packed cell volume (PCV) Blood samples of about 3-5 ml were collected from Jugular/wing vein of each diseased bird in a separate sterilized test tube containing sufficient volume of, Anticoagulant "1ml of 1% EDTA" in 5 ml of blood for the purpose of hematological studies. Blood smears were prepared by placing and spreading a small drop of blood at cleaned surface of microscopic glass slides spreader smears then dried, labeled, stained by Geimsas method and was checked by , Digital Optika Microscope's Model. no. B3-4083 Italy (Bhatti et al., 1989).

4. RESULTS

During present research 72 containing population (96,500) broiler chickens were thoroughly surveyed blood samples were collected from the Jugular/ wing veins of naturally infected broilers with Hydro pericardium syndrome and apparently healthy broilers of the same age groups of different poultry flocks around Hyderabad during the entire study period. The blood samples were subjected to (a) total erythrocyte count (b) total leukocyte count (c) hematocrit values (d) erythrocytic sedimentation rate and (e) haemoglobin percentage. The hematological values of different commercial broiler chickens of 3-5 weeks of age affected with hydro pericardium syndrome (HPS).

Table.1. demonstrated that, the mean total erythrocytic count (TEC) was 2.228±0.139 millions/cmm in broilers affected with leukocytic count recorded as 14.5±2.296 in infected flocks. The mean haemoglobin count 6.820±0.327 grams/100ml. Erythrocytic sedimentation rate (ESR) 3.458±0.291 mm/hr. and packed cell volume (PCV) 26.482±0.817 percent were recorded at random in broiler flocks infected with hydro pericardium syndrome (HPS) respectively.

The mean total erythrocytic count (TEC) in normal broilers of various flocks was recorded at random showed 3.11±0.484 millions/cmm. Similarly the mean total Leukocytic count (TLC) was 25.820±0.884 thousands/cmm. Haemoglobin (HB) 10.890±0.427 grams/100ml of blood, erythrocytic sedimentation rate (ESR) 2.340±0.089 and packed cell volume 30.540±0.650 percent, recorded at random in normal broilers of various flocks.

Table.2. revealed that, the mean neutrophils 38.880±1.890 percent, eosinophils 1.004±0.097 percent, Basophils 0.734±0.067 percent, lymphocyte 55.480±2.132 percent and monocytes 3.340±0.270 percent in blood samples; taken at randomly from the infected broiler chickens with (HPS). The mean neutrophils were 33.880±0.311 in percent apparently normal broilers among all different flocks. The mean value of eosinophils was 1.496±0.204 percent, basophils 0.878±0.055 percent, lymphocytes 69.360±0.152 percent and monocytes 4.660±0.598 percent were found in apparently normal broiler birds of different flocks.

Table.1. Hematological values among affected and non-affected broilers with Hydro-Pericardium Syndrome.

S.N 0	Parameter	Non-affected birds	Affected birds	
		Mean ± SD	Mean ± SD	Probability
1	TEC (Million/ cmm)	3.11± 0.484	2.228± 0.139	0.0043
2	TLC (Thousand/ cmm)	14.500± 2.298	25.820± 0.844	0.0000
3	HB (%) (gm/ 100ml)	10.890± 0.427	6.820± 0.327	0.0000
4	ESR (mm/Hr.)	2.340± 0.089	3.458± 0.291	0.0000
5	PCV (%)	30.540 ± 0.817	26.482± 0.817	0.0000

Mean Values show significant difference

Table.2: Differential leukocytic count among affected and non-effected broilers with Hydro Pericardium Syndrome.

S. No	Parameter	Non-affected birds	Affected birds	
		Mean ± SD	Mean ± SD	Probability
1	Neutrophils	22.880± 0.311	38.880±1.800	0.0000
2	Eosinophils	1.496± 0.204	1.004± 0.097	0.0012
3	Basophils	0.878± 0.055	0.734± 0.067	0.0060
4	Lymphocytes	69.360± 0.152	55.480±2.132	0.0000
5	Monocytes	4.660± 0.598	3.340± 0.270	0.0020

Mean Values show significant difference

5. DISCUSSION

Hydro pericardium syndrome (HPS) or "Angara disease" is a mysterious disease particularly affecting chickens of 3-5 weeks of age. Hydro pericardium was first reported in commercial broiler flocks at Angara goath near Karachi, Pakistan during August and September, 1987 and gradually spread to the adjoining areas of Karachi (Jaffery et al., 1986). Hydro pericardium syndrome (HPS) inflicts heavy economic losses in millions of rupees and mortality ratio reaches up to 70% and gives constant threat to the poultry industry (Jaffery et al., 1986, Khan et al., 1988, Khan et al., 2005, Khan et al., 2012). The present research was undertaken to record the hematological variation among commercial broiler breeds.

Blood values of broilers infected with hydro pericardium syndrome, the erythrocytes, leukocytes and haemoglobin

concentration were decreased in affected broiler chickens. Among leukocytes, neutrophils were showing marked increase with a relative decrease of lymphocytes, monocytes and eosinophils in ailing broilers. The present study demonstrated significant decrease with exception of leukocytes that, was similar to the finding of (Jaffery et al., 1988, Kumar et al., 2003, Balamurugan and Kataria. 2004) who recorded increase in number of leukocytes along with decrease in lymphocytes and eosinophils in sick birds but was partly coincided to the finding of (Anjum et al., 1989, Khan et al., 2005, Peters et al ., 2011) who observed the certain changes in hematological values.

The significant decrease in the mean total of erythrocytic count, hematological content and hematological values which were detected in broilers affected with hydro pericardium syndromes (HPS) during entire period of the study were in agreement with the finding of (Bhatti et al., 1989, Borrego and soto. 1995, Rani et al., 2011, Tehrani et al., 2012), who recorded the similar changes in affected broilers with simple variation in blood values which could be due to severe destruction and haemolysis resulted from viral infection in infected broilers.

6. CONCLUSION

It is concluded from current study that, the hydro pericardium syndrome affects the hematological values of broiler chickens; however strict preventive measures should be taken to avoid infection.

DATA statistical analysis M stat - computer software package.

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Sequence Analysis of PreS2 Region of Hepatitis B Virus Genotype D Isolates

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ABSTRACT

Hepatitis B virus (HBV) is a well known agent of liver diseases. HBV disease burden varies across the globe with regions from low to high endemicity. Pakistan lies in the intermediate endemic zone, with high rate of mortality due to liver disease, cirrhosis and hepatocellular carcinoma. There is a wide range of heterogeneity in relation to HBV genotypes and sub-genotypes and in their patterns of pathogenesis, virulence and response to antiviral therapy. A large number of HBV genomic variations are associated with clinical outcomes such as hepatocellular carcinoma and liver cirrhosis. Thus, the present study aims to analyze PreS2 gene sequences from HBV isolates and their phylogeny. To investigate this, a study was conducted on twenty one HBV chronically infected individuals, serum samples were subjected to PCR with specific primers for PreS2 region of HBV genotype D and then sequenced. Point mutations: A39V, P41H and L42I were found in cell permeability domain of PreS2 protein. However, MHC class I and II epitopes were conserved in all sequences. Phylogenetic analysis was carried out by comparing the nucleotide sequence with 22 reference sequences of HBV sub-genotype D retrieved from the GeneBank. Phylogenetic analysis showed that two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2.

Key words: Hepatitis B virus; Phylogenetic analysis; PreS2 region; Genotype D; Pakistan

1. INTRODUCTION

Hepatitis B virus (HBV) infection is a threat to the public health worldwide. Incidences of HBV infections are increasing at an alarming rate due to unavailability of effective vaccines and improved antiviral therapies for low income population. According to the current estimates of World Health Organization (WHO) 400 million people are chronically infected worldwide (Locarnini, 2002). In Pakistan, there are estimated 7-9 million carriers. Recently, a study reported 28.87% and 22% in HBV positive patients suffering from liver cirrhosis and hepatocellular carcinoma respectively Ali et al. (2011).

On the basis of genetic variability of (i.e.> 8%), HBV can be classified into eight genotypes including genotypes A to H Okamoto et al. (1988). All genotypes exhibit distinct geographical distributions, depicting ethnographic patterns of disease transmission Kramvis et al. (2005). Further, these eight genotypes have been grouped into 49 sub-genotypes on the basis of nucleotide variability of 4% to 8% Schaefer et al. (2005). In Pakistan, Genotype D (63.71%) is the most prevalent genotype. Moreover, sub-genotypes D1 and D3 have been reported to be prevalent in Pakistani population Baig et al. (2009). In addition, presence of D2 has also been reported Ahmed et al. (2009). However, Genotype C prevails with 7.55% in local population and is considered as an emerging genotype Ali et al. (2009).

HBV is a member of Hepadnaviridae family Pungpapong et al. (2007). The virus genome is double stranded circular virus of 3.2 kilobase pairs, which is organized in four overlapping open reading frames ORF S, C, P and X. ORF S encodes surface protein. ORF C encodes either HBV e antigen or viral capsid protein that has the ability of self assembly into the viral capsid. ORF P and ORF X encode large polymerase protein and 16.5 kDa HBV X protein respectively. ORF S is divided into three in frame AUG start codons which encodes three different surface proteins (Schadler and Hildt, 2009). Large surface protein (LHBs) encompasses PreS1 (108-119aa), PreS2 (55aa) and Surface (226aa) domains. Middle protein (MHBs) includes PreS2 and surface domain. Moreover, Small protein (S) consists of only Surface domain.

PreS2 domain consists of human serum albumin binding site (aa3-16), N-glycosylation site (aa 4), transactivator domain, cell permeability domain (aa 41-52) Lin et al. (2012) and several T and B cell epitopes Barnaba et al. (1989). Numerous mutations have been reported in PreS2 gene mutations which add complication in the diagnosis and treatment of HBV infected individuals (Weber, 2006; Gao et al., 2007; Su et al., 2007). In addition, PreS2 mutations are also associated with clinical outcomes. Point mutations; L36P, A39V, L42I, P41H, P52R were detected in Occult HBV isolated from patients with Hepatocellular Carcinoma Pollicino et al. (2007).

There is insufficient data regarding the mutations in the PreS2 region in local HBV genotype D infected individuals. Therefore; this study aimed to isolate PreS2 ORF gene and analyze the PreS2 sequence for mutations. In addition, a phylogenetic analysis was conducted to determine origin of PreS2 origin of HBV genotype D isolates.

2. MATERIALS AND METHODS

Blood Sample collection and Viral DNA extraction

A total of 21 blood samples of HBV positive patients were collected from patients residing in Islamabad during January to April, 2012 at Atta-ur-Rahman School of Applied Biosciences's Diagnostics Lab. Consent of the patients was taken and they were informed about the research study. Out of 21 samples only three samples corresponded with the inclusion criteria. Accordingly, samples having HBV infections other then genotype D and either Human immunodeficiency virus or Hepatitis C virus infections were excluded from the study; demography of the samples is shown in Tabel 1. Total of 3ml of freshly drawn blood was taken in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and centrifuged at 8000 rpm for 3 minutes to separate the serum. Genomic DNA was purified from the serum by using Nucleospin kit (Germany) according to the manufacturer's protocol. Primers were designed by retrieving the already published sequences of HBV PreS2 gene of genotype D from National Centre for Biotechnology Information (NCBI) (Available at http://www.ncbi.nlm.nih.gov/). The PreS2 gene was amplified by the following primers: TGTAGGCCCACTCACAGCA (forward) and GTAACACGGCAGGGGTCCT (reverse).

Table 1. Demography of patients

Accession no.	Sex	Age	HBsAg	HBeAg
ASAB 1(2266)	M	41	POSITIVE	POSITIVE
ASAB 2(HF2)	F	18	POSITIVE	POSITIVE
ASAB3(PIMS7)	M	33	POSITIVE	POSITIVE

PCR Amplification of HBV PreS2 gene and Sequencing

Polymerase Chain Reaction (PCR) was carried out to amplify PreS2 gene using the extracted Genomic DNA. The reaction mixture was prepared in 0.2 ml tubes (Axygen, California, USA) by adding 12.5 µL (800 ng) sample DNA template along with 2.5 µL each of gene specific forward and reverse primer (2 pmol), 5 µL of 2 mM dNTPs ,5 µL of 25 mM MgCl2 , 1.25 µL of 1.5 units of Taq polymerase (1U/µL) (Fermentas, USA) along with 5 µL of 10X Taq Polymerase Buffer and nuclease free water (16.25 µL) making a total reaction volume of 50 µL. PCR mixture was vortexed and then placed in Thermal Cycler (Swift™ MaxPro Thermal Cycler, Esco, Singapore), under the following PCR conditions: 94° C for 2 min followed by 35 cycles of 94° C for 1min, 58.5° C for 45 s, 72° C for 60 s and a final extension at 72° C for 10 min. Reactions was held at 4° C. Amplified PCR products were analyzed by electrophoresis on 1 % agarose gel. DNA fragments were purified from agarose gel by using Silica Bead DNA Gel Extraction Kit (Fermentas, USA). Purified DNA products were sent to Macrogen Korea for sequencing along with 10µl of forward HB2250P3F and HB200P3R reverse primer.

Sequence and Phylogenetic Analysis

PreS2 isolates were sequenced from each patient by using both sense and antisense primers. These sequences were aligned in CLC workbench software (CLC Inc, Aarhus, Denmark) to draw a consensus sequence for each isolate and then consensus sequences were submitted to Genebank under accession number KF482899, KF482900, KF470787. Sub-genotype D sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) : AY741798-IRAN D1, AY161159-INDIA D1, AY796032-TURKEY D1, AB583680.1-PAKISTAN D1, FJ562309.1-CHINA D1, FJ386590.1-CHINA D1, JN642163.1- LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2 JN642163.1- LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2, EI00615-EAST INDIA D3, X85254-ITALY D3,AB583679.1-PAKISTAN D3, AY373430-INDIA D3, JF815648.1- BRAZIL D4, GQ922005.1-CANADA D4, GQ922003.1-CANADA D4, GQ205386.1-INDIA D5, GQ205387.1-INDIA D5, JF815661.1-BRAZIL D6, JF815606.1-BRAZIL D6, FJ904444.1-TUNUSIA D7, FJ904440.1-CHINA D7, shown in Table 2. Consensus sequences were generated for sub-genotype D1 to D7. Then subsequent consensus and our PreS2 isolates sequences were aligned using CLC workbench 6.5.3 (www.clcbio.com). Further CLC tool was used to translate nucleotide sequence into protein sequence. B and T cell epitopes, along with other protein domains were analyzed. Phylogenetic analysis of our three PreS2 isolates with PreS2 sequences of sub genotype D was performed by CLC workbench software.

3. RESULTS

Nucleotide Sequence Alignment of PreS2 gene

A total of 22 Sub-genotypes (D1-D7) of genotype D HBV PreS2 sequences were retrieved from Genebank as shown in Table 2. PreS2 sequences for every genotype D sub-genotypes were aligned and a consensus sequence was generated. Consensus sequences were then compared with our isolates: ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7). Point mutations at C28T, T125C and C122A were found in PreS2 sequences as shown in Figure 1, which were further analyzed via protein alignment.

Protein Sequence Alignment of PreS2 protein

Amino acid sequences were predicted for ASAB1 (2266), ASAB2 (HF2), ASAB3 (PIMS7) and PreS2 consensus sequences using CLC Work Bench 6.5.3. Later, amino acid sequences were aligned. Mutations were found in cell permeability domain of three of our isolates as shown in Figure 2. In ASAB1 (2266) and ASAB2 (HF2) isolates, ALA at position 39 is substituted with VAL, where both are neutral and non polar in nature. Moreover, in all three isolates at position 42, LEU is replaced by another neutral and non polar amino acid ILE. However, PRO at position 41 which is neutral and non polar, is substituted by HIS a basic and polar amino acid.

Phylogenetic analysis

Phylogenetic tree was constructed by using three different PreS2 gene sequences of HBV reported in this study along with twenty two PreS2 sequences obtained from Genebank

with different sub-genotypes. Hepatitis B virus sub-genotypes were grouped in individual clusters. Two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2 as shown in Figure 3.

Table 2. Retrieved PreS2 sequences with sub-genotype of D from NCBI

SUB-GENOTYPE	NCBI ACCESSION NO-ORIGIN
D1	AY741798-IRAN, AY161159-INDIA, AY796032-TURKEY, AB583680.1-PAKISTAN, FJ562309.1-CHINA, FJ386590.1-CHINA
D2	JN642163.1- LEBANON, EU594382.1-RUSSIA, JF815677.1-BRAZIL
D3	EI00615-EAST INDIA, X85254-ITALY, AB583679.1-PAKISTAN, AY373430-INDIA
D4	JF815648.1- BRAZIL, GQ922005.1-CANADA, GQ922003.1-CANADA
D5	GQ205386.1-INDIA, GQ205387.1-INDIA
D6	JF815661.1-BRAZIL, JF815606.1-BRAZIL
D7	FJ904444.1-TUNUSIA, FJ904440.1-CHINA

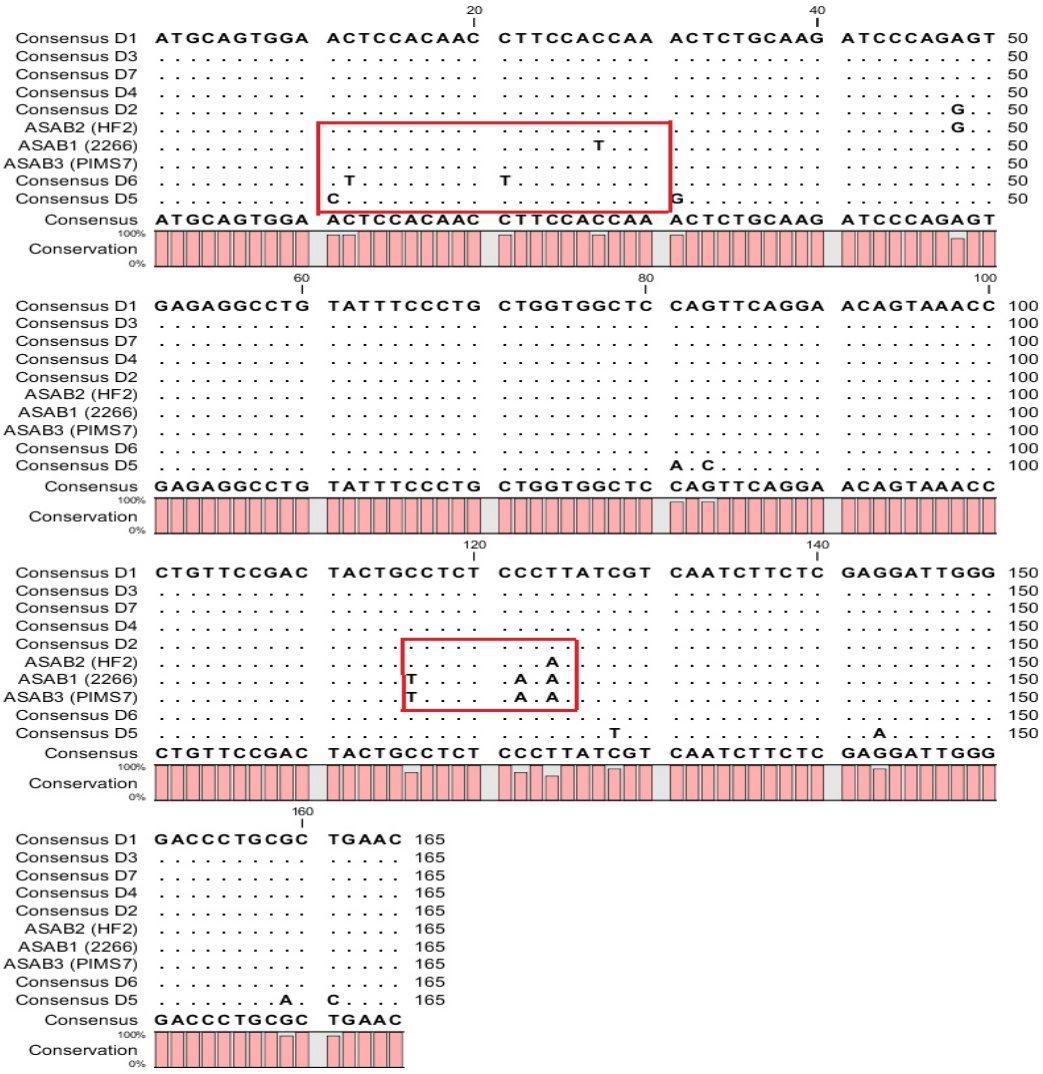

Figure 1. Multiple sequence alignment (MSC) of HBV PreS2 gene sequences of sub-genotype D strains with ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7) isolates by means of CLC workbench 6.5.3 (http://www.clcbio.com).

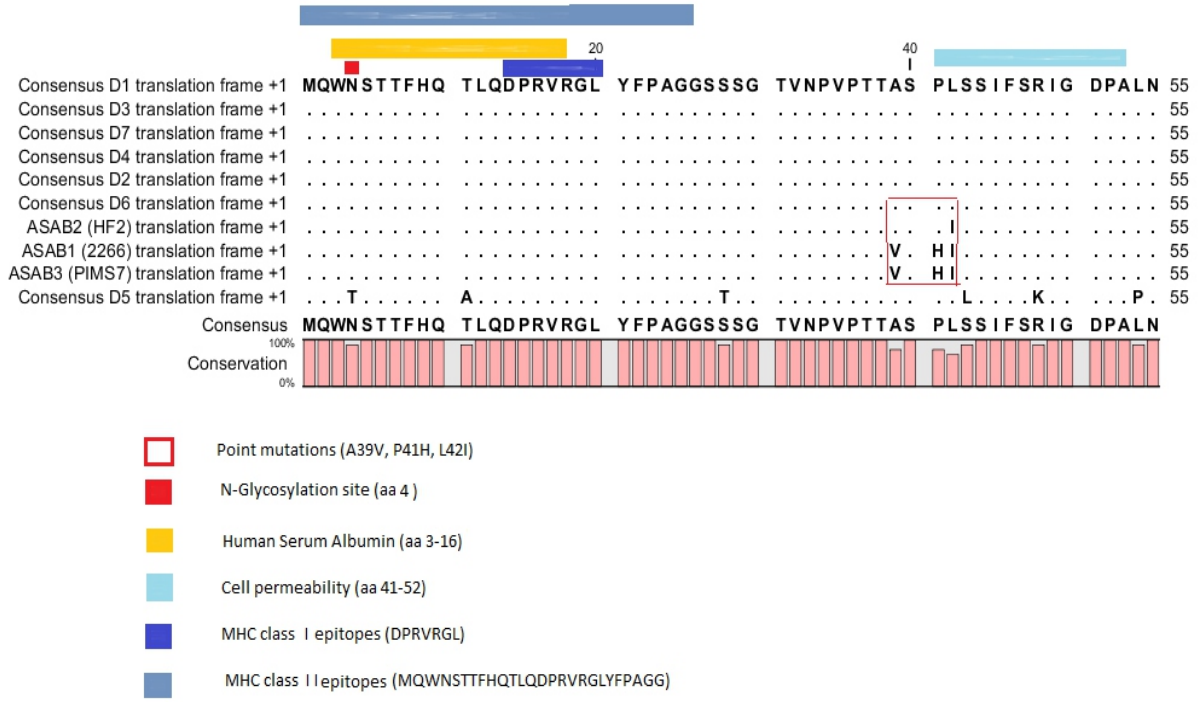

Figure 2. Protein sequence alignment of 3 of our isolates with other protein consensus sequences of sub-genotype D mentioned in Table 2. The conserved bases are shown as dots whereas the mutated residues are marked with the single letter code of that amino acid. Colored squares represent respective domains in the PreS proteins (Lin et al., 2012) .Used software CLC workbench 6.5.3 (http://www.clcbio.com).

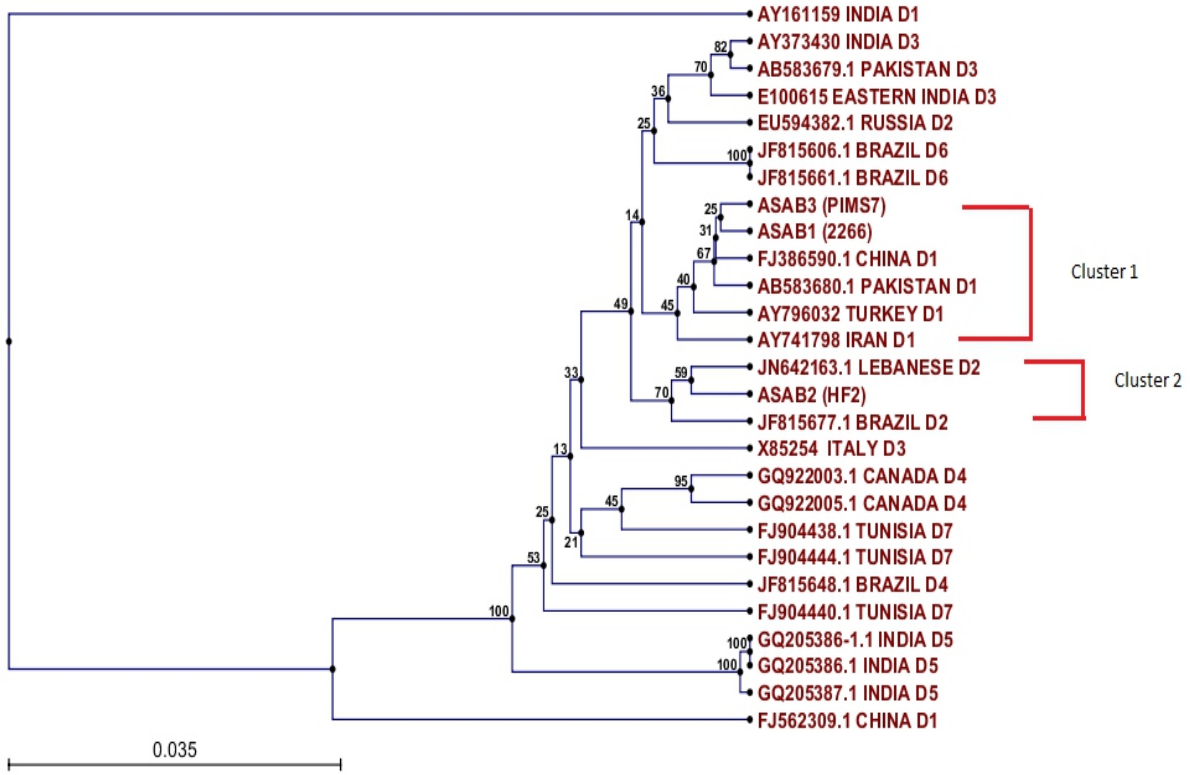

Figure 3. Phylogenetic tree of HBV PreS2 gene sequences. Tree was constructed by UPGMA algorithm. Bootstrap values are mentioned at the nodes. Tree shows a phylogenetic relationship between three of our isolates and sub-genotypes from the rest of the world. References sequences are labeled by Genebank accession number, Country name and by their respective sub-genotypes.

4. DISCUSSION

In Pakistan, the prevalence of HBV infections has increased beyond the reported findings. There are estimated 7-9 million carriers with carrier rate of 3-5%. In Pakistan, HBV infected population is distributed among general population including healthy blood donors, military recruits, prisoners and healthcare persons Ali et al. (2008). There are 20 to 30% HBV infected patients with cirrhosis and hepatocellular carcinoma Ali et al. (2011). Thus, HBV is a real challenge for scientist community.

HBV mutants have substantial impact to trigger pathogenesis and cause severe form of liver disease Mendy et al. (2008). Deletion mutations spanning 42-54 bp within Pre-S2 N-terminal, Pre-S2 internal deletions: 1-15 PreS2, 1-26 PreS2, 12-20 PreS2, 44-53 PreS2 have been reported (Gao et al., 2007; Su et al., 2007). Where, PreS2 internal deletions lead to the loss of M protein-glycosylation site (Asn-4 glycan site) in PreS2 protein, which impairs virion secretion (Margaret and Reinhild, 1998). Further, deletions in PreS2 region alter T and B cell epitopes giving PreS2 mutants selective advantage over wild type viruses Chisari et al. (1995). Studies report, HBV PreS2 deletion mutants confer an inefficient immune response, hence PreS2 deletion mutants persists in infected individual Barnaba et al. (1989). HBV Isolates from Ground glass Hepatocytes showed deletions at 5 terminus of PreS2 region (nt 2-55) and point mutation within the start codon (ATG to ATA) of middle surface protein (Fan, 2000). Recent studies found, prevalence of 18.8% Pre-S2 deletions in the HCC group and 5.8% PreS2 deletions in non-HCC group. In addition, PreS2 deletions were more prevalent in HCC patients aged < 50 years as compared with older HCC patients Yeung et al. (2011). Thus, keeping in view this scenario, study was conducted to determine PreS2 variants. PreS2 gene was successfully isolated and sequenced from three genotype D patients suffering from HBV chronic infections. Moreover, protein sequence derived from nucleotide sequences were analyzed for mutations.

Point mutations ALA to VAL, LEU to ILE and PRO to HIS were found, these have been previously reported in occult HBV isolates from patients with hepatocellular carcinoma Pollicino et al. (2007). These mutations were present in cell permeability domain which is important for translocation of viral protein and nucleic acids Hildt et al. (2002). Several MHC epitopes are reported in the entire HBV envelope protein Barnaba et al. (1989). Moreover, MHC class I and MHC class II epitopes were conserved in all sequences. Humoral and cellular immune response is directed against HBV envelope protein which leads to viral clearance, and neutralizing activity of anti-HBs antibodies. So far, only small protein (S) has been extensively used for the preparation of the vaccines and production of antibodies for therapeutic purposes. However recently, Madalinski et al. (2004) demonstrated that the third-generation preS1/preS2/S vaccine containing PreS2 major histocompatibilty epitopes showed more rapid onset and pronounced antibody response as compared to the S-gene-derived vaccine in healthy children and newborns (Madalinski et al., 2004; Madalinski et al., 2001). Thus, third generation vaccines can be used with further validation whether PreS2 MHC epitopes are conserved in local HBV variants.

Moreover, recently several researches demonstrated that type of chronic outcome; prevalence of mutations and the severity of virulence vary for genotypes and sub-genotypes. Thus, for better disease management and prognosis, it is imperative to determine their global distribution. Several studies report distribution of HBV sub genotypes around the globe. Where, Genotype A is further categorized in sub genotypes A1 predominant in Asia and Africa, A2 in Northern Europe and America (Bowyer et al., 1997; Kramvis et al., 2002) and A3 (Mulders et al., 2004; Kurbanov et al., 2005) in Central Africa. Sub-genotypes B1 of genotype B dominates in Japan, whereas B2 in China and Vietnam (Sugauchi et al., 2004; Norder et al., 2004). Genotype C with C1 as sub-genotype is common in South-East Asia and Bangladesh, C2 in Japan, Korea, and China (Chan et al., 2005; Sugauchi et al., 2004), C3 in Oceania and C4 in Australia (Sugauchi et al., 2003; Norder et al., 2004). In addition, geographical distribution of sub-genotypes D designated as D1–D7 are found to be in India, Iran, and Indonesia.

In this study, a phylogenetic analysis was conducted for three of our isolates. ASAB1 (2266) and ASAB3 (PIMS7) were grouped in cluster 1 and shared common ancestors with other members of the clusters, hence belong to sub genotype D1. While, ASAB2 (HF2) showed sequence homology with sub genotype D2 in cluster 2.

5. CONCLUSION

In conclusion, mutations were detected in PreS2 cell permeable motif. However, the implication of mutations in this region needs to be further elucidated by in silico protein modeling. Phylogenetic study demonstrated two sequences from sub-genotype D1 and one from sub-genotype D2. By further increasing number of PreS2 sequence analysis of HBV infected patients a considerable statement can be made about different mutations associated with sub-genotype.

6. ACKNOWLEDGEMENTS

We thank Dr Hajra Sadia for providing us valuable suggestions during the course of the study. This work was supported by Pak-US Science and Technology cooperative program entitled “Hepatitis B Virus-Associated Hepatocellular Carcinoma in Pakistan”.

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Exploring the Protein Interactome Related to Hepatitis C Virus

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ABSTRACT

Hepatitis C virus (HCV) stands as a health problem experienced across the globe leading to chronic or acute liver diseases such as cirrhosis, hepatocellular carcinoma and various others. It is a complex disease with extensive genetic heterogeneity with little known about the interactions of complex intra- and intercellular processes. The evolving tools in the application of network science to identify diseases have paved a way for the study of complex diseases at system level. This study focuses on identifying the significant proteins and the biological regulatory pathways involved in Hepatitis C virus and performing topological analysis of the PPIs derived by the proteins encoded by the susceptible genes in order to look for the molecular connectivity between these pathways.

Key words: Protein-protein interaction, network, regulatory pathways, analysis, construction of PPI and Hepatitis C Virus

1. INTRODUCTION

Hepatitis Inflammation of the liver is an infection which causes chronic liver diseases such as cirrhosis and is widely linked to hepatocellular carcinoma. The infection may be found in an acute or chronic form. Successful treatments are available for Hepatitis C through antiviral drugs but the chronicity of the disease may lead to a prolonged liver damage. Hepatitis can be classified in to five main types namely, Hep C (HCV), Hep A (HAV), Hep B (HBV), Hep E (HEV) and Hep D (HDV). All of these types are responsible for liver damage with few discrete differences (Sun et al., 2015).

Hepatitis C virus (HCV) belongs to the Flaviviridae viral family with a positive, single-strand RNA molecule as a genome with extensive genetic heterogeneity. It has an open reading frame responsible for encoding a large polyprotein of about 3000 amino acids . It transmits through infected blood, and becomes the most serious type of hepatitis amongst the others, consequently becoming the global health problem infecting approximately 200 million people around the globe (Isken et al., 2007).

Currently no vaccination is available for HCV and therapies have been failed to improve the health of patients suffering with disease (Pawlotsky, 2006).

According to Centre for disease control (CDC), in 2009 there were about 16,000 reported cases of acute HCV out of which about 60 to 70 percent are likely to develop chronic liver disease . Chronic infection leads to liver cirrhosis in approximately 30% of infected individuals (McDermott et al., 2012).

Protein–protein interactions (PPIs) establish physically between more than two proteins as a consequence of biochemical events and/or electrostatic forces. At cellular and systemic levels proteins are macromolecules of dynamic nature, but they seldom act alone. PPIs are an essential part of biological processes. Therefore, the construction of PPI networks provides a foundation for understanding protein function. The unidentified protein function can be predicted on the basis of their PPIs, whose function is already revealed (Rao et al., 2014).

In the recent years, high-throughput methods have been advanced in order to measure the transcript or protein levels, globally at system level. These methods are used to identify genes or proteins that are likely to be involved in a diseased process -in this case HCV, in order to direct further experimental investigation. Since the disease process consists of proteins of fundamental importance, the PPIs encoded by the susceptible genes were considered to be significant in the pathogenicity of HCV. Furthermore, recent advances in topological analysis have been applied to interactome networks comprising PPI networks, where physical interactions depicts nodes . This study is intended to recognize the significant proteins and the biological regulatory pathways involved in HCV pathogenicity and perform topological analysis of the PPIs derived by the proteins encoded by the susceptible genes in order to look for the interactions between these pathways at molecular level.

2. METHODS

The presented study comprised six steps which are as follows:

2.1. Manual Screening of Proteins Associated with HCV from the Literature

The first step included manual screening of the proteins associated with HCV by using the PolySearch online text mining system, producing genes/proteins related to "Hepatitis C virus" by analyzing multiple sources of information which includes PubMed, OMIM, Swiss-Prot and DrugBank. Other

than the gene/proteins, it covers concepts like diseases, drugs, pathways, Single Nucleotide Polymorphisms, metabolites and tissues . "Disease-Gene/protein association" query type and the query keyword "Hepatitis C Virus" were used. Initially the system returned 499 literatures which were further manually screened. Finally, 294 candidate genes were obtained by checking their relevance to HCV from the published resources and eliminating the genes that weren't related to HCV (Supplementary Information).

2.2. Scanning for Protein-protein Interactions

The candidate genes obtained in step one was used to scan PPIs in step two. In order to scan the PPIs, they were extracted from STRING database (http://string-db.org/), which is a source for discovering protein-protein interactions.

2.3. Construction of the PPIs Network

An extended network was constructed consisting obtained proteins and their protein-protein interaction (PPI) neighbors along with the interactions between these proteins. In order to construct this network a highly resourceful tool –Pajek, was used . One giant network and two separate small networks –consisting of four proteins were obtained from the extended network. Through this information, the study directed the discovery of HCV at system level, and due to the high number of nodes in the giant network large betweenness centrality (BC) values were expected to be in that parameter. Considering this fact, only the giant network was thoroughly studied.

2.4. Topology of PPI Network

The analysis of PPI network topology involves major properties of nodes which form the basis of the network analysis or process. Hence, to evaluate the nodes in a network, connectivity degree (k), BC and closeness centrality (CC) were implemented. Degree and BC are two basic parameters in the network theory , . Degree (k) is the interactions between a protein and its neighbors and is the fundamental property of a node. Closeness centrality (CC) points out the center of the network, as it is the inverse of the mean length of the shortest paths directing to and from all the other nodes in the graph. Betweeness centrality (BC) is the measure of nodes that occur on the shortest paths amongst other nodes i.e. number of shortest paths that pass through each node. BC is a highly useful property as it indicated the detection of bottleneck proteins in a network. Also, it greatly influences over what flows in the network. Furthermore, average degree, mean shortest path length and diameter are some of the measures of topology used globally to character network . Average degree (<k>) is the average of all degree values of nodes present in a network. Mean shortest path length (mspl) connects each pair of nodes via shortest path by calculating average of the steps. And, the longest amid all shortest paths is diameter (D). All these characteristics were used to characterize the network using Pajek.

2.5. Creation of Backbone Network

A backbone network is made up of high BC proteins and the links between them. In order to create a backbone, 10% of the total node set was set as high BC to get maximum number of nodes as the backbone in order to study the genes associated with HCV i.e. 146 in the network. Hence, the first 15 BC nodes and their connections were extracted from the giant network to extract a backbone network. In network, BC was initially used to calculate the centrality of the nodes. The shortest paths function as bottleneck to control the communication between other nodes in the network as they go through the nodes with high BC.

2.6. Construction of Betweenness Centrality (BC) Values

A subnetwork of the genes associated with HCV, either connected indirectly or directly with the shortest path between the genes, was constructed. This was done by measuring the shortest paths using Pajek, resulting in a subnetwork including nodes present in these paths.

3. RESULTS

3.1. Protein-protein Interaction Network

The extended network comprises one large network along with two separated small networks which resulted from four seed proteins, POLR2K, ITPA, IL28B and GPT2 (Figure 1). The large network comprised 146 nodes. The backbone network consisted of 25 nodes. The largest degree in the giant network is 44, whereas the average degree is 9.630.

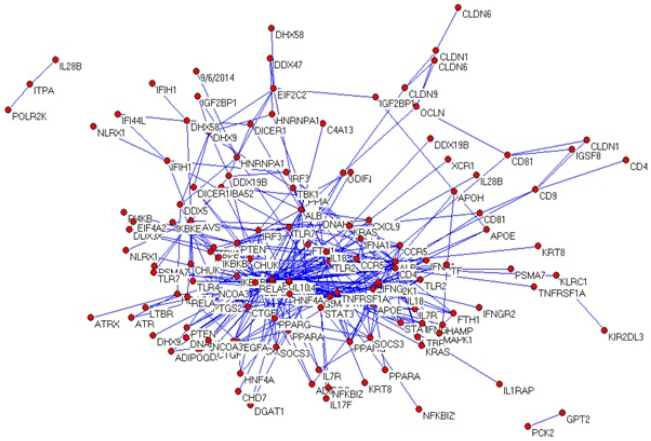

Figure 1. Extended network. The network comprises one large network along with two small networks resulting from four seed proteins, POLR2K, ITPA, IL28B and GPT2.

3.2. Key Nodes Identification

In this study, the key nodes were classified as the ones with high BC value or large degree, and 10% of the 146 nodes were used as the integral point of high BC and large degree nodes. Of 146 nodes, 15 have highest betweenness centrality (Table 1), 15 have high degree (Table 2) and 15 nodes were selected with large degree and high BC values (Table 3). Also, 4 proteins (CD4, VEGFA, MAVS, IFNG) were selected amongst the 15 nodes with BC value only (also mentioned in Table 3). So, to distinguish the different nodes and their roles in the network, different colors were assigned to each category (Figure 2). Pajek returned values calculated from the network for the degree, BC and CC value. MAPK1 (Mitogen-activated protein kinase 1) is a hub (center) protein with the largest degree, while CD4 is a bottleneck protein with the highest BC. Whereas, DIF is the central protein in the network

with the most interactions since it has the highest CC.

Table 1. The high betweeness centrality nodes along with their CC values

S. No.	Symbol	BC	CC value
1.	CD4	0.146060821	0.429224
2.	MAPK1	0.115570473	0.403598
3.	MAPK1	0.115570473	0.403598
4.	RELA	0.085469027	0.407245
5.	ALB	0.08177989	0.390767
6.	STAT3	0.077300083	0.396497
7.	ALB	0.070137334	0.385201
8.	PPARG	0.061925174	0.378726
9.	VEGFA	0.060126883	0.414741
10.	UBA52	0.056967454	0.414741
11.	TLR4	0.053361921	0.407245
12.	MAVS	0.050364462	0.325013
13.	IFNG	0.049459971	0.3919
14.	EIF2C2	0.046524672	0.281092
15.	TLR2	0.044640386	0.394185

Table 2. The large degree nodes along with their CC values

S. No.	Symbol	Degree	CC value
1.	MAPK1	44	0.403598
2.	RELA	43	0.407245
3.	DIF	38	0.441847
4.	CD4	38	0.429223
5.	STAT3	34	0.396497
6.	UBA52	33	0.414740
7.	TLR4	32	0.407245
8.	TNFRSF1A	32	0.385200
9.	MAVS	31	0.325013
10.	IFNG	30	0.391899
11.	PPARG	29	0.378726
12.	TLR2	28	0.394185
13.	TBK1	27	0.347571
14.	VEGFA	26	0.414740
15.	RXRA	26	0.318880

Table 3. List of high BC and large degree node along with their functions

Symbol	Function Description
MAPK1	Acts as a point that integrates various biochemical signals
RELA	REL-associated protein involved in the formation of NF-C heterodimer and the activation and translocation in the nucleus.
DIF	A dorsal-related gene found in Drosophilia, responsible for mediating an immune response.
CD4	Glycoprotein found in the immune system on the surface of T helper cells, monocytes, macrophages, and dendritic cells
STAT3	It directs the production of proteins which are involved in the pathways responsible for chemical signaling within the cells.
UBA52	A very conserved protein found in the nucleus and cytoplasm, that targets the cellular proteins for degradation
TLR4	Involved in recognizing pathogens and activating innate immunity
MAVS	Encodes an intermediary protein essential in the signaling pathways of beta interferon, triggered by virus
IFNG	Soluble cytokine with eminent properties like antiviral, immunoregulatory and anti-tumor and plays a role in activating macrophages
PPARG	Belongs to the nuclear receptor subfamily i.e. peroxisome proliferator-activated receptor (PPAR). PPARs play a vital role in formation of heterodimers with retinoid X receptors (RXRs) and regulate transcription of numerous genes
TLR2	Involved in recognizing pathogens and activating innate immunity
VEGFA	This protein is a glycosylated mitogen that specifically acts on endothelial cells and has effects like: mediation of increased vascular permeability, angiogenesis induction, cell growth of vasculogenesis and endothelial, cell migration promotion, and apoptosis inhibition

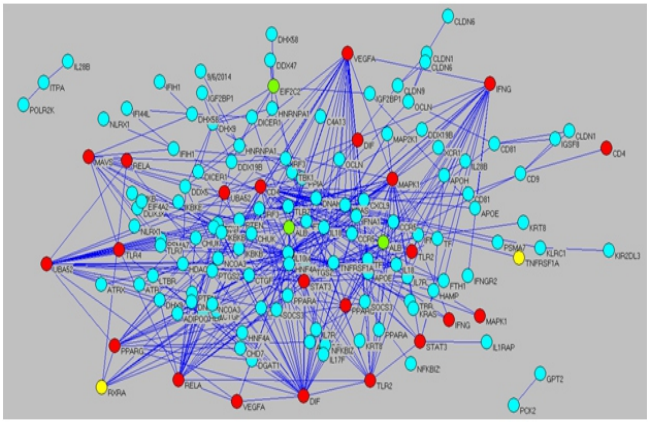

Figure 2. Giant network topology.

3.3. Cross-talk Between the Signaling Pathway in the High BC Network and Backbone Network Derived from them

A backbone network was constructed with high BC nodes. There 15 high BC nodes present in the backbone network

which corresponded to their sizes. DIF is the center protein and has 10 neighbors (Figure 3): CD4, ALG, PPARG, VEGFA, RELA, TLR4, STAT3, UBA52, IFNG and MAPK1. These proteins are involved in Leishmaniasis, Pancreatic cancer, Dorso-ventral axis formation, processing and presentation of antigen, T cell receptor signaling pathway, autophagy regulation and many more.

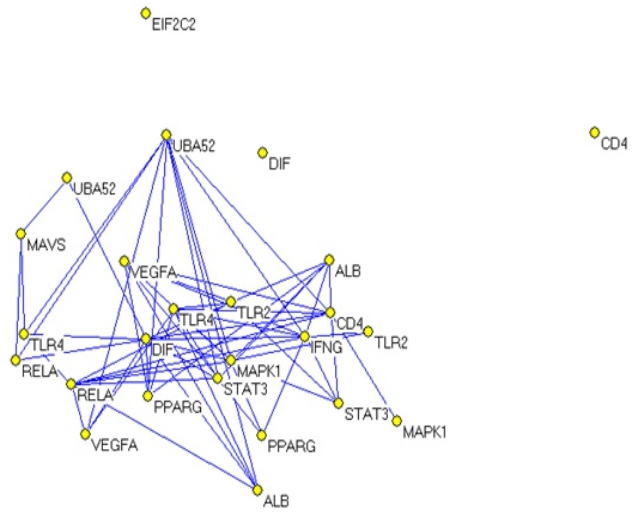

Figure 3. Backbone network topology.

3.4. Subnetwork Between the Candidate Genes Consisting of all Shortest Paths

The subnetwork consists of 146 nodes comprising 15 proteins that are not high BC nodes, 15 large BC nodes, and 116 seed proteins (Figure 4). It can be discovered that CD4 has the largest BC value and that the top 15 BC nodes in this network correspond with the backbone network nodes. There are only 15 proteins not in the list of 5 nodes with large BC value in the giant network. They are IL28B, C4A13, CHD7, PCK2 and POLR2K (Table 4).

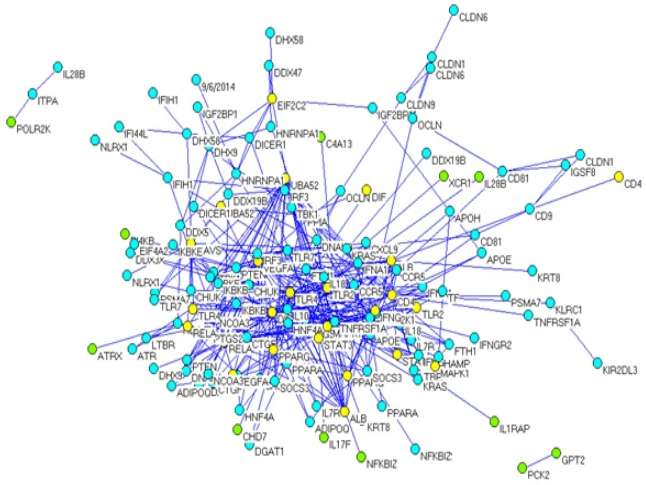

Figure 4. The subnetwork. It includes the paths shortest amongst the genes relevant to HCV. All shortest paths connect the candidate genes (yellow color nodes). There are 15 nodes that are without large BC (green color)

Table 4. The list of top 15 BC nodes in the subnetwork

S. No.	Symbol	BC
1.	CD4	0.146060821
2.	MAPK1	0.115570473
3.	DIF	0.103334907
4.	RELA	0.085469027
5.	ALB	0.08177989
6.	STAT3	0.077300083
7.	ALB	0.070137334
8.	PPARG	0.061925174
9.	VEGFA	0.060126883
10.	UBA52	0.056967454
11.	TLR4	0.053361921
12.	MAVS	0.050364462
13.	IFNG	0.049459971
14.	EIF2C2	0.046524672
15.	TLR2	0.044640386

4. DISCUSSION

Although a number of studies report the numerous genes relevant to HCV, it's pathogenesis still has room for further study. This study focuses on the analysis of how the proteins found contribute to the pathogenesis of HCV and determines other key proteins through topological analysis. The evaluation of proteins in PPIs related to the disease is done by the two fundamental properties of network theory i.e. degree and betweenness.

146 genes have been searched in this study as genes susceptible to HCV. The network constructed from the converted seed proteins, consists of a large giant network and two small networks (Figure 1). Five seed proteins (POLR2K, ITPA, IL28B, PCK2, and GPT2) included in the two separated networks indicate HCV variation between these proteins. As proposed by Ran et al. , at times some genes are missed from the literature search and new genes susceptible to the disease may be discovered. Hence, false interactions may lead to false recognition of nodes. MAPK1 with the highest degree is on 2nd rank in large BC proteins list. On the other hand, CD4 with largest BC is ranked 4th in the high degree proteins list.

The bottle neck proteins included UBA52, a very conserved protein found in the nucleus and cytoplasm, that targets the cellular proteins for degradation MAVS gene encodes a fundamental protein essential for the virus-triggered beta interferon signaling pathways . Protein encoded by TLR4 gene is a member of the Toll-like receptor (TLR) family including TLR2 gene is involved in recognizing pathogens and activating innate immunity . IFNG gene belongs to type II interferon family. It is a soluble cytokine with eminent properties like antiviral, immunoregulatory and anti-tumor and plays a role in activating macrophages. PPARG gene belongs to the nuclear receptor subfamily i.e. peroxisome

proliferator-activated receptor (PPAR). PPARs play a vital role in formation of heterodimers with retinoid X receptors (RXRs) and regulate transcription of numerous genes. VEGFA gene found as a disulfide linked homodimer, is a member of the growth factor family PDGF/VEGF. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has effects like: mediation of increased vascular permeability, angiogenesis induction, cell growth of vasculogenesis and endothelial, cell migration promotion, and apoptosis inhibition . DIF (Dosral-related Immunity Factor) is a well-studied gene in Dorsophila Melanogaster. DIF gene is normally localized in cytoplasm of larva responsible for mediating an immune response. . DIF gene is involved in the differential activation of NF-kappaB. In nucleus DIF binds to sequence motifs like kappa B found in promoter regions of genes responsible for immunity . This protein is the center protein because of its highest CC value.

4.1. Differential Activation of NF-kappaB by DIF

HCV infection initiates innate antiviral responses comprising the production of IL-28A, IL-28B, and IL-29, known as type III interferon. But, the molecular mechanisms involved in expression regulation of IFN genes in hepatocytes infected with HCV remains unclear. The binding of specific transcription factors to promoter regions was further determined by the regulatory elements inducing the IFN genes resulting in hepatocytes infected with HCV. Interferon regulatory factor (IRF) -3 and -7 are the transcriptional factors required for the stimulation of interferons IL-28A and IL-28B genes, while NF-B required for the stimulation of the IL-29 gene. A decrease in viral replication was observed by the addition of IFN- to HCV-infected hepatocytes resulting in microRNA-122 (miR-122) reduction .

The family of NF-KappaB (Nuclear Factor-kappa B)/I-KappaB stimulates the expression of over a 100 proteins contributing in the host immune response. The target proteins comprise cytokine and chemokine receptors essential for immune recognition, antigen presentation proteins, and adhesion receptors. NF-KappaB has been labeled the central mediator of the immune response, because of this extensive role in immune action.

Multiple families of viruses, including HCV (Hepatitis C Virus) activate NF-KappaB. This activation may have several functions: to promote viral replication, prevent virus-induced apoptosis, and mediate the immune response.

5. CONCLUSION

Amongst the 146 candidate genes, most of them were associated with HCV and their protein-protein interaction neighbors connected to a large network. The backbone network gave an evident overview of all the important genes in HCV. The finding suggested the link between HCV and the PPI network which was centered at DIF. DIF gene is involved in the differential activation of NF-kappaB which in turn is relevant to HCV which activates this particular factor.

6. ACKNOWLEDGMENT

Administration of Mohammad Ali Jinnah University, Islamabad is acknowledged for providing the necessary facilities for the study. The authors have full access to all of the data in the study and take responsibility for the integrity of the data and accuracy of the analysis.

Conflict of Interest

The authors declare no conflict of interest related to this manuscript.

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Pharmacogenomics of Type 2 Diabetes Mellitus; A Step Toward Personalized Medicine

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ABSTRACT

Type 2 diabetes is a complex multifactorial disease characterized by insufficient insulin secretion and insulin resistance. Global prevalence of diabetes is increasing day by day making diabetes a global epidemic. Various factors increase risk of diabetes and genetic predisposition is one of very important factor. Many anti-diabetic treatments are available to control blood glucose level in diabetic patients now-a-days. Initially, oral anti-diabetic treatment is successful but it fails later on and requires insulin administration and there are large numbers of non-responders also. Even patients that do respond show variability in drug response and tolerance. Pharmacogenomics is the study to determine the inter-individual differences that contribute toward drug response. Many studies have shown positive contribution of different polymorphism in alteration of drug response in various ethnicities. But still data related to pharmacogenomics of Asian population is limited. In this review, we have tried to summarize the genetic variations and their effect on three major classes of oral anti-diabetic medication that include thiazolidinediones, metformin and sulfonylureas.

Key words: T2D, pharmacogenomics, anti-diabetic, sulfonylurea, thiazolidinediones, metformin

1. INTRODUCTION

Type 2 diabetes (T2D) is a common chronic disease that is characterized by hyperglycemia and insulin resistance. It is associated with various co-morbidities like hypertension, obesity, hyperlipidemia, nonalcoholic fatty liver disease and resulting microvascular and macrovascular complications (neuropathy, retinopathy, atherosclerosis, nephropathy, and cardiovascular disease — . Prevalence of diabetes is increasing globally and recent studies show that underdeveloped countries in continent like Asia and Africa are more affected than developed countries like United States. It is estimated that about 371 million people suffered from diabetes in 2012 and it will increase to 552 million people till 2030 . T2D is also a disease of development and lifestyle changes. The known risk factors for diabetes include modifiable (overweight and obesity, dietary factors, sedentary lifestyle) and non-modifiable risk factors (age, ethnicity, sex, history of gestational diabetes and family history) (Chen et al., 2012).

It has been known for more than five decades that genetic differences among people contribute toward differences in response to drugs. Pharmacogenomics is the term used for studying inter-individual genetic differences that influence the drug response. It is a relatively new field of study that involves identification of genes and inter- and intra-individual variations that not only affect response to drug but also help to design and predict new drugs. Similarly like other diseases, the genetic variability among individuals also influences anti-diabetic treatment that contributes toward metabolism, absorption and distribution of drugs. These genetic variations might also affect the drug target making some individual drug resistant. Identification of such variations related to drug response can help physicians in making drug selection decisions, better disease management, avoiding adverse drug reactions (Hu, 2012).

Along with the various known environmental factors that increase the risk of type 2 diabetes, multiple genetic factors also contribute toward its pathogenesis through interaction with detrimental environmental factors . Recently using the candidate gene studies and genome-wide association studies more than 40 loci have been associated with T2D among common variants in European genome . T2D is a complex disease so these genetic associations do not identify the exact causal variant and culprit gene for pathogenesis of T2D (Ostbye et al., 1989; Zeggini et al., 2008; Billings and Florez, 2010; Dupuis et al., 2010; Voight, Scott et al., 2010; Herder and Roden, 2011; Saxena et al., 2013). These genetic variations contribute toward the pharmacodynamics and pharmacokinetics of drugs because various genes encode proteins that are involved in drug absorption and metabolism (Huang and Florez, 2011). Primary management of T2D involves lifestyle modifications, but these may not be enough. As the disease progresses T2D management likely will require medication. Previously only two oral medicines were available, but recent advances in therapeutics have provided physicians with an opportunity to choose from various medicines with different modes of action including sulfonylurea, biguanides, a-glucosidase inhibitor, thiazolidinediones, glinides, DPP-IV and SGLT2 inhibitors, and non-insulin injectable (Goldfine, 2001; Gadsby, 2002). Their mechanism of action includes increasing insulin secretion (sulphonylureas and meglitinides), reduction of carbohydrate uptake from gastrointestinal tract and decreasing level of glucose release from liver (Metformin, thiazoledinediones, and alpha glucosidase inhibitors),

however the biological mechanisms of some anti-diabetic medication are not well understood (Goldfine, 2001; Huang and Florez, 2011).

1.1. Thiazolidinediones

Thiazolidinediones are a class of drugs that increase insulin sensitivity in the liver, muscle, and fat tissues. These agents increase lipolysis and suppress glucose release from liver through increased binding of the peroxisome proliferator-activated receptor γ (PPARG) to its target (DNA response element), thereby decreasing the glycemic load on the pancreatic beta cell (Hofmann et al., 1992; Saltiel and Olefsky, 1996; Altshuler et al., 2000). Initial studies on pharmacogenetics were focused on a common polymorphism of PPARG (functional missense mutation P12A). This mutation has been reported to be associated with protective effect against T2D (Altshuler et al., 2000).

One study showed a significant decrease in glucose levels in people carrying this P12A polymorphism in response to rosiglitazone administration (Bozkurt et al., 2007), but two other studies (Bluher et al. 2003; Snitker et al., 2004) showed that percentage of responders does not differ between alanine carriers and proline homozygotes. In one of these studies, the TRIPOD study (Troglitazone in prevention of diabetes), one third of the cases did not show an increase in insulin sensitivity in response to troglitazone. Snitker et al. continued their studies by genotyping the 131 common variants of PPARG and found association of eight different polymorphisms in response to troglitazone administration. Conversely, Kang et al. showed that patients with P12A polymorphism showed better response to rosiglitazone treatment. Another study failed to show an association of P12A along with five other polymorphisms with troglitazone (Bluher et al., 2003; Snitker et al., 2004; Kang et al., 2005; Wolford et al., 2005; Florez et al., 2007). One possibility for these variable results might be presence of other novel variants in PPARG or other T2D associated genes influencing response to thiazolidinediones. Environmental and epigenetic factors can also influence drug response. However, the use of troglitazone was withdrawn from market due to its hepatotoxic effects (Fowler, 2007).

In contrast carriers of P12A show an increase in glucose levels, resulting in higher conversion to diabetes in response to acarbose. This effect has also been shown in carriers of a polymorphism in adiponectin gene (ADIPOQ) (Bozkurt et al., 2007). A polymorphism in CYP2C8 gene was found to be associated with changed clearance rate of rosiglitazone (Kirchheiner et al., 2006). All of these results need to be verified through extensive experiments and research.

1.2. Biguanide

Metformin is a safe and effective medicine that primarily increases glucose uptake in muscle and fat tissues and reduce gluconeogenesis output from liver thus increasing insulin sensitivity similar to thiazolidinedione's (Montanari et al., 1992; Bell and Hadden, 1997). It is the first line biguanide agent in treatment of T2D (Stumvoll et al., 1995). The absorption of metformin from intestine involves two steps. The first step involves active uptake process that is mediated by broad-specificity transporters OCT1 and OCT2 that belong to highly polymorphic solute carrier family 22 (SLC22A1 and SLC22A2) to hepatocytes and renal tubular cells, respectively. The second step involves the MATE1 protein (multidrug and toxin extrusion protein) that helps excretion of un-metabolized metformin into bile and urine. OCT 2 has more affinity than OCT1 for metformin. A study in Chinese and Japanese population found that nonsynoymous polymorphisms in SLC22A1 were associated with different plasma concentration of metformin (Koepsell, 1998; Jonker and Schinkel, 2004; Kimura et al., 2005; Koepsell et al., 2007; Shu et al., 2008; Chen et al., 2010).

Metformin stimulates AMP-activated protein kinase (AMPK), a key regulator of glucose metabolism. Metformin induces AMPK activation or its adenosine analogue suppresses SREBP-1 expression (which is an important transcription factor of lipogenesis). Research has shown that activated AMPK is required for inhibitory effect of metformin on glucose production by hepatocytes (Zhou et al., 2001). A GWAS in Scottish patients in response to metformin treatment showed an association of rs11212617 SNP in ATM gene with metformin treatment success. Variation in ATM gene upstream of AMPK modifies response to metformin. This indicates that ATM and AMPK both genes enhance metformin response (Zhou et al., 2011). Association of this SNP has been replicated in Netherland and UK (van Leeuwen et al., 2012). Another study showed that ATM gene does not directly affect AMPK activation in response to metformin. Instead KU-55933 reduces AMPK activity independent of ATM (Woods et al., 2012). Another study also showed role of KU-55933 in metformin response (Yee et al., 2012).

Different SLC22A1 variants cause increase or decrease in uptake of metformin. Studies have shown that variants of SLC22A1 including Gly220Val, Ser189Leu, Ser14Phe, Met420del, Arg61Cys, Gly401Ser, and Gly465Arg increase uptake and variants Arg61-Cys, Ser189Leu, Gly220Val, Gly401Ser, Met420del, Gly465Arg decrease uptake in vitro (Shu et al., 2008). Polymorphisms Arg61Cys, Gly401Ser, Met420-del, and Gly465Arg with synonymous variant Ser52Ser were found to be associated with renal clearance of metformin by reduction in OCT1 expression/activity (Chen et al., 2010). A European cohort study suggested association in reduction of HbA1c in diabetic patients with non-coding SNP rs622342C/A in response to metformin treatment, but this study was not replicated in American-European population (Becker et al., 2009; Jablonski et al., 2010).

SLC22A2 variants Thr201Met and Ala270Ser were identified in a small Japanese group of non-responders to metformin treatment, and a new variant Thr199Ile was identified in Korean population. These variants influence clearance and tubular excretion of metformin and thus increase plasma concentration of metformin (Kang et al., 2007; Shikata et al., 2007; Song et al., 2008; Wang et al., 2008; Chen et al., 2009). However, association and genotype-phenotype correlation for these variants could not be replicated in Asian populations (Sakata et al., 2004; Kirchheiner et al., 2006; Kim et al., 2007; Shu et al., 2008; Jablonski et al., 2010; Huang and Florez, 2011).

1.3. Sulfonylureas

Sulfonylureas is a class of T2D medication that binds to the sulfonylurea receptor SUR1 in the plasma membrane of the beta cells that is coupled to the KATP-channel. KATP-channel consists of two subunits Kir6.2 and SUR1 and triggers glucose dependent stimulation of insulin secretion (Panten et al., 1996; Gloyn et al., 2004). These subunits are encoded by potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11) gene and ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (ABCC8) gene, respectively (Flanagan et al., 2009). Studies have shown gain of function and loss of function mutations in these genes cause neonatal diabetes mellitus. These mutations generate permanent opening or closure of KATP channels causing insulin deficiency or hypersecretion of insulin, respectively (Gloyn and Ellard, 2006; Murphy et al., 2008; Sattiraju et al., 2008). These mutations' effects suggest that these could be potential target to study drug responses.

Sesti et al. showed that patients with lysine 23 (K23) variant of the KCNJ11 glutamic acid (E) 23K polymorphism had a relative risk of secondary failure (loss of effective anti-diabetic response after years of treatment) 1.45 as compared with homozygotes of E23E (Sesti et al., 2006). This study is in contrast to UK Prospective Diabetes Study (UKPDS) in which significant association of 23K was not found in response to sulfonylurea treatment (UK Prospective Diabetes Study Group 1998). Later, various meta analysis studies showed strong association of E23K polymorphism of KCNJ11's association with response to sulfonylurea treatment (Florez et al., 2004; Holstein et al., 2009). These studies in Caucasian populations did not show any association with higher risk of secondary sulfonylurea failure in patients (Gloyn et al., 2003;Sesti et al., 2006; Holstein et al., 2009). Also a decreased sulfonylurea response was observed in isolated human pancreatic islets from diabetic donors carrying KCNJ11 E23K variant (Sesti et al., 2006). Two other studies in Asian populations showed better response to sulfonylurea therapy in patients having risk allele of both KCNJ11 and ABCC8 (Zhang et al., 2007; Feng et al., 2008). Despite previous studies saying what, a 2012 study showed strong association of E23K variant of KCNJ11 in response to sulfonylurea treatment for first time in caucasions (Javorsky et al., 2012).

KCNJ11 and ABCC8 genes are located on chromosome 11 and separated by a region of only 5kb, which is why many variants within these genes are strongly correlated (Florez et al., 2004). A missense mutation Ala1369Ser (A1369S) in ABCC8 is strongly associated with KCNJ11 E23K in all populations studied (Inoue et al. 1997; Florez et al. 2004). Hamming et al., showed that K23/A1369 variant increases the risk of T2D, sunsequently associating the A allele in ABCC8 with increased risk of T2D and enhanced reponse to gliclazide in vitro (Hamming et al., 2009). Various studies have showed strong association of variants of ABCC8 in the UK, Denmark, Neitherland, France, Mexican, and Caucasian cohorts from Utah in the USA, Chinese and American populations (Inoue et al., 1996; Hani et al., 1997; Goksel et al., 1998; Hansen et al., 1998; Hart et al., 1999; Elbein et al., 2001; Meirhaeghe et al., 2001; Barroso et al., 2003). Meta analysis of ABCC8 gene variants rs1799854C/T and Thr759Thr (T759T) showed no association in caucasian poulation (Gloyn et al., 2003). The variants in ABCC8 and KCNJ11 were not associated with response to glimepride treatment in Korean population. This contradiction in reported study results suggests the hypothesis that the risk haplotype of KCNJ11 and ABCC8 gene causes a conformatonal change in the sulfonylurea binding domain recognized by some sulfonyurea molecules (Cho et al., 2011; Lang et al., 2012).

The strongest association of any gene reported to be associated with T2D to date is for TCF7L2. This association has been reported in a substantial number of ethnicities. Although the exact mechanism of how TCF7L2 contributes toward pathogenesis of T2D is still not clear, it possibly causes a defect in GLP-1 metabolism (Damcott et al., 2006; Florez et al. 2006; Chandak et al., 2007; Loos et al., 2007; Wang et al., 2007). Various replication studies also confirmed its association with decreased sulfonylurea treatment response (Cauchi et al., 2006; Florez et al., 2006; Grant et al., 2006; Humphries et al., 2006; Pearson et al., 2007). Go-DARTS study of UK population showed that T allele variant of TCF7L2 is associated with therapeutic failure in T2D patients (Kimber et al., 2007). Another study carried out by the same group showed that patients with risk allele failed treatment with sulfonylurea, but had no effect on metformin treatment (Pearson et al., 2007). This association was confirmed by a study conducted in German population which also showed increased rate of sulfonylurea tretment failure in patients having T allele in TCF7L2 gene at rs7903146 (Holstein, Hahn et al., 2011). In Chinese population another intronic variant rs290487C/T was reported that influence eficacy of reaglinide in patients with T2D (Yu et al., 2010).

These studies suggest that genetic variations play an importanct role not only in the pathogenesis of complex diseases like T2D but also influence the drug metabolism and action. Ethnic variations also play an important role in disease susceptibility and it is important to study genetic variations associated with diseases in multiple ethnicities and compare the differences and the effect of these variations on ethnicities as well.

2. CONCLUSION

Common oral anti-diabetic therapies include anti-hyperglycemic drugs that are administered immediately after diagnosis. These oral anti-diabetic drugs include thiazolidinediones, metformin, sulfonylureas, dipeptidyl peptidase-IV (DPP-IV) inhibitors and GLP1 mimetics. It is clear from clinical data that initially diabetic patient's glycemic control is well managed by anti-diabetic treatment but with the passage of time combination therapy may be required. Ultimately anti-diabetic treatments fail to control diabetes and thus require insulin administration as well. There is also variable response to oral anti-diabetic treatment due to inter-individual variability. Reserchers are struggling to identify underlying genetic variance that modify drug reponse but to date very limited informations is available and only few genes and polymorphisms have been verified to be associated with anti-diabetic therapy. These findings are also not consistent among ethnicities and even less data is available for

Asian populations. Due to complex nature of disease it is not possible to infer complete mechanism of modified drug reponse by one SNP. For this purpose new techniques like next generation sequencing should be employed to sequence all genes that are associated with T2D to find their role and effect in drug metabolim. Specially additional research is needed in Asian countries to generate comprehensive data. Despite all the efforts and positive results we cannot apply pharmacogenetics in clinical practice unless accurate interpretation of genetic factors of anti-diabetic therapeutic response has been achieved.

Conflict of Interest

The authors declare no conflict of interests

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Cloning and Partial Characterization of Cotton Leaf Curl Burewala Virus From Khanewal

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ABSTRACT

Begomoviruses are a serious threat to cotton production throughout the world. In Pakistan, enormous crop losses occur as a result of cotton leaf curl disease (CLCuD) caused by begomoviruses. Molecular characterization of begomoviruses has made possible the identification and analysis of begomoviruses prevalent in a host plant. Infected cotton leaf sample (C-59) was obtained from area around Khanewal during 2011. The total DNA was isolated from the infected sample by Cetyl trimethyl ammonium bromide (CTAB) method. An expected size band of approximately 1100bp, covering coat protein region of the virus, was amplified using universal primers. The amplified product was T/A cloned and sequenced to its entirety. DNA sequence showed 99% nucleotide sequence identity to each of Cotton leaf curl Burewala virus ((CLCuBuV; Accession No HF549184)) and Cotton leaf curl Kokhran virus (CLCuKV; Accession No AJ002449)). Since CLCuBuV is a recombinant of CLCuKV and Cotton leaf curl Multan virus and the coat protein region of CLCuBuV was derived from CLCuKV that is most probable reason that the available sequence showed identity with CLCuBuV as well as CLCuKV. A complete characterization of full length virus will determine whether isolate C-59 is CLCuBuV or CLCuKV. Literature indicates that there is no existence of CLCuKV within the region and CLCuBuV is dominating within Indo-Pak.

Key Words: Cotton leaf curl Burewala virus, Cotton leaf curl Multan virus, Cotton leaf curl Kokhran virus, Cotton leaf curl disease, C5, Coat protein, Replication enhancer protein.

1. INTRODUCTION

1.1. Begomoviruses

Begomoviruses have been divided into two groups based on genome organization. The genome is either monopartite or bipartite. Monopartite genome constitutes only DNA A whereas bipartite genome is composed of DNA A and DNA B components. The genome replication takes place through double stranded DNA intermediates (Hanley-Bowdoin et al., 1999).

1.2. Bipartite Begomoviruses

Bipartite Begomoviruses have two genomic components designated as DNA A and DNA B (Lazarowitz, 1992). Six genes are coded by DNA A whereas DNA B codes two genes. Coat protein gene (CP or V1) and the pre-coat protein (V2) gene are encoded on the virion sense strand whereas replication-associated protein gene (Rep or C1), transcriptional activator protein gene (TrAP or C3), replication enhancer protein gene (REn or C3) and the C4 gene are found on the complementary sense strand of DNA A. Movement protein (MP) and the nuclear shuttle protein (NSP) are coded on the DNA B component (Kallender et al., 1998).

1.2.1. Function of the Virion Sense Proteins

The coat protein plays a role in encapsidation of ssDNA, movement of the begomovirus and insect transmission (Sharma and Ikegami 2009). Silencing suppression and pathogenicity determination is carried out of by the pre-coat protein (Yadava et al., 2010).

1.2.2. Function of the Complementary Sense Proteins

Located in the IR is the origin of replication containing a nonanucleotide sequence (TAATATT/AC) that forms a stem loop structure. Binding of Rep to the viral iterons positioned upstream of the origin of replication present in the IR initiates viral DNA synthesis (Nagar et al., 1995). Transcriptional activator protein (TrAP) blocks hypersensitive response to the begomovirus infecting a plant (Mubin et al., 2010). It also acts as a silencing suppressor and functions in the up-regulation of virion sense genes (Yang et al., 2007). Replication enhancer protein (REn) is involved in viral DNA replication (Pasumarthy et al., 2011). The C4 protein is involved in the determination of symptoms and virus movement (Jupin et al., 1994). All these six proteins are encoded on the DNA A component (Figure 1.1).

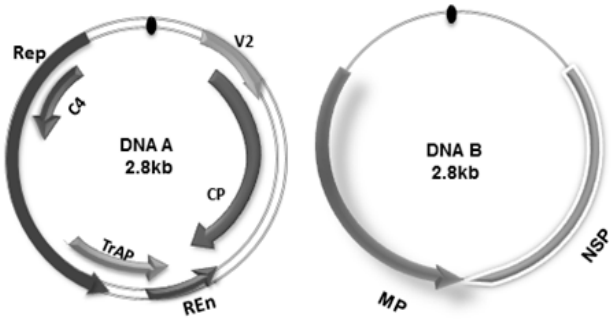

Figure 1.1. Genomic maps of DNA A and DNA B components of a begomovirus.

1.2.3. Function of the Proteins on DNA B

Movement protein (MP) aids in the movement of the bipartite begomovirus across plasmodesmata. The virus is transported from the nucleus to the cytoplasm with the help of nuclear shuttle protein. Both proteins are encoded on the DNA B component (Hanley-Bowdoin et al., 1999) (Figure 1.1).

1.3. Monopartite Begomoviruses

A sole genomic element of approximately 2.8 kb termed as DNA A constitutes the genome of monopartite begomoviruses. This single genomic constituent holds genes that are responsible for all viral functions in its entirety (Tan et al., 1995). Some monopartite begomoviruses require a betasatellite for their symptom modulation. The size of the betasatellite is half the size of the helper virus and depends on it for the encapsidation, replication, insect transmission and movement. Betasatellite shares no sequence similarity with the helper component although the nonanucleotide sequence is conserved (Zhou et al., 2003). (Figure 1.2).

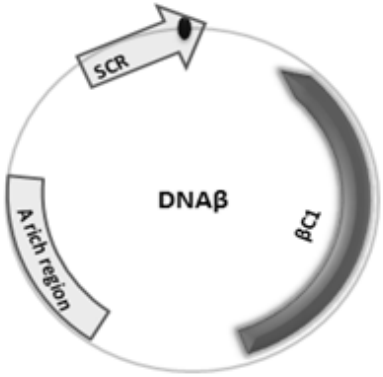

Figure 1.2. Betasatellite molecule identified in begomoviruses.

1.3.1. Function of Betasatellites

The only gene encoded by the begomovirus betasatellite is the βC1 gene, encoded on the complementary sense strand, required for the virulence of the helper begomovirus. A region rich in adenine and a satellite conserved region (SCR) are located on the virion sense strand (Rojas et al., 2001) (Figure 1.2). Betasatellites can be trans-replicated by any helper component in a non-specific way. The best example is the trans-replication of Cotton leaf curl Multan betasatellite (CLCuMB) by Ageratum yellow vein virus (AYVV) (Saunders et al., 2008).

1.4. Importance of Begomoviruses and Cotton Leaf Curl Disease

The devastating losses of crops such as tomato, bean, cotton and cucurbits in USA at the end of 20th century due to the dissemination of begomoviruses by Bemisia tabaci have been reported (Henneberry et al., 2000) Pakistan economy has suffered due to losses in cotton production as a result of Begomovirus infection with additional losses in cucurbits, cassava and pepper production (Briddon and Markham, 2000). The major disease affecting cotton in Pakistan since 1967 is the cotton leaf curl disease (CLCuD) first reported from Multan. It is a disease complex that involves leaf curling and formation of enations on the underside of leaves. (Hussain et al., 1991). The disease in Pakistan occurred in 1967 in the Punjab province with a decline in the disease incidence at the end of the 20th century due to the establishment of resistant cotton varieties against it. The disease has emerged as a serious threat to cotton production since 2001 due the development of overpowering breakdown of resistance in the disease causing begomovirus. Until 2003 occurrence of CLCuD in Sindh was irregular with no serious consequences to the cotton production in Sindh than in other localities of Pakistan but in the year 2003 the disease has appeared to be severe and devastating (Mansoor et al., 2003).

2. MATERIALS AND METHODS

Infected cotton leaf samples were collected from area around Khanewal during 2011. The leaves were stored at -80°C until further processing.

2.1. Diagnosis of the Viral DNA

The method employed for the isolation of total nucleic acid was similar to that set forth by Doyle and Doyle in 1990. The extracted DNA was subjected to polymerase chain reaction using universal primers (Table 2.1).

Table 2.1. Primer pairs for partial amplification of begomoviruses.

Primer	Sequence
CLCV1	CCGTGCTGCTGCCCCCATTGTCCGCGTCAC
CLCV2	CTGCCACAACCATGGATTCACGCACAGGG

2.2. Transformation and Cloning

The PCR product run on 1% agarose gel was gel eluted using Silica Bead DNA Gel Extraction Kit # K0153. Ligation of the purified DNA with the pTZ57R/T vector was performed (InsTAcloneTM PCR Cloning Kit). Competent E. coli DH5α cells were prepared using calcium chloride method as mentioned by Cohen et al. (1972). Transformation of competent E. coli DH5α cells was followed using standard protocol as mentioned by Sambrook and Russell, 2001.

2.3. Screening of Potential Clones

Plasmid DNA was isolated using Alkaline lysis method. Double digestion of the isolated plasmid with 5 units each of EcoR1 and HindIII was performed to confirm the presence of insert. Plasmid isolated using kit protocol (FavorPrepTM Nucleic acid extraction) was sent for sequencing.

3. RESULTS

3.1. Diagnosis of the Viral DNA

PCR amplification of the extracted DNA using universal primer designed for the partial characterization of begomovirus revealed a fragment of approximately 1.1kb on 1% agarose gel (Figure 3.1).

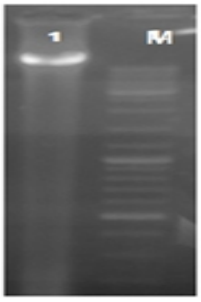

Figure 3.1. Total DNA (T. DNA) extracted using CTAB method. Lane 1: Contains T. DNA of C-59. Lane M: Standard 1kb ladder.

3.2. Transformation and Cloning

The purified DNA was ligated into T/A cloning vector (pTZ57R/T) vector for transformation. The ligated product was used to transform DH5@ cells. Blue white colonies appeared on the agar plate containing ampicillin, IPTG and XGAL after 16 hours incubation. White colonies were selected for the screening of clones containing the required DNA fragment.

3.3. Screening of Potential Clones

Double digestion of the isolated plasmid with 5 units each of EcoR1 and HindIII generated two fragments: one fragment of approximately 3000bp or 3kb and the other of nearly 1200bp or 1.2kb. The larger band indicated the plasmid DNA while the small band indicated that the partial amplified DNA had been cloned (Figure 3.2).

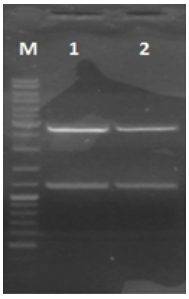

Figure 3.2. Gel picture showing double digestion of Plasmid DNA with EcoR1 and Hind111. Lane 1 shows double digestion of plasmid DNA obtained for clone 1. Lane 2 shows the double digestion of plasmid DNA obtained for cone 2.

3.4. Sequence Analysis

3.4.1. Sequence Analysis Using NCBI BLAST and ORF Finder

Nucleotide BLAST results showed 99% sequence identity with the isolate CLCuBuV-[PK-Okara: 11] (Accession number: HF549184) and CLCuKV-Fai [PK: Fai1] (Accession number: AJ496286).

Sequence analysis was done using open reading frame finder (ORF finder). Three partial ORFs were found in C-59. In frame amino acid number of each protein translated form the partial ORFs were predicted and compared with that in the full length isolates (Table 3.1) and amino acid sequence identity is shown in Table 3.2.

Table 3.1. Predicted ORFs based on nucleotides.

ORFs in C-59	Predicted number of nucleotides in C-59	Position of ORFs in a typical begomovirus
CP	378 – 1062	292 - 1062
Ren	1059 -1211	1059 - 1463
C5	378 – 807	283 - 807

Table 3.2. Amino acid sequence identity.

ORFs	Predicted number of amino acids in C-59	Predicted Amino acids	Predicted number of amino acids in matched isolates	Percent identity
CP	208	256	Cotton leaf curl Kokhran virus (Accession number: NC_004583)	99%
REn	50	134	Cotton leaf curl Burewala virus (Accession number: AM774301)	98%
C5	161	175	Cotton leaf curl Burewala virus (Accession number: FN645929)	99%

3.4.2. Construction of Sequence Map

The sequence map shows that the C5 gene and the CP gene overlap with each other but in different orientation. Some part of REn overlaps with the CP gene. The CP ORF is present in the virion sense strand while REn ORF is located on the complementary sense strand.

3.5. Phylogenetic Analysis

The phylogenetic dendrogarm based on multiple sequence alignments between partial sequence obtained in the present study and with sequences available in the database showed its presence between CLCuBuV and CLCuKV (Figure 3.3).

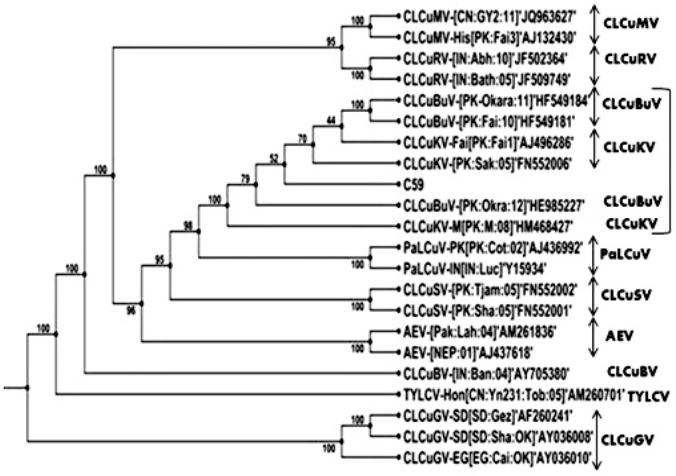

Figure 3.3. Phylogenetic tree generated using CLC sequence viewer. Cotton leaf curl Multan virus-Hisar[Pakistan: Faisalabad 3] (Accession number: AJ132430), Cotton leaf curl Mulatn virus -[China:GY2:2011] (Accession number: JQ963627), Cotton leaf curl Rajhasthan virus-

[India:Bathinda:2005] (Accession number: JF509749), Cotton leaf curl-[India:Abohar:2010] (Accession number: JF502364), Cotton leaf curl Burewala virus-[Pakistan:Faisalabad:2010] (Accession number: HF549181), Cotton leaf curl Burewala virus-[Pakistan:Okra:2012] (Accession number: HE985227), Cotton leaf curl Burewala virus-[Pakistan-Okara:2011] (Accession number: HF549184), Cotton leaf curl Gezira virus-Egypt[Egypt: Cairo: Okra] (Accession number: AY036010), Cotton leaf curl Gezira virus-Sudan[Sudan: Shambat: Okra] (Accession number: AY036008), Cotton leaf curl Shadadpur virus-[Pakistan:Shahdadpur:2005] (Accession number: FN552001), Cotton leaf curl Shadadpur virus-[Pakistan:Tandojam:2005] (Accession number: FN552002), Papaya leaf curl virus-India[India: Lucknow] (Accession number: Y15934), Papaya leaf curl virus-Pakistan[Pakistan:Cotton:2002] (Accession number: AJ436992), Ageratum enation virus-[Nepal:2001] (Accession number: AJ437618), Ageratum enation virus-[Pakistan:Lahore:2004] (Accession number: AM261836), Tomato yellow leaf curl virus-Honghe[China: Yunnan231:Tobbaco:2005] (Accession number: AM260701), Cotton leaf curl Kokhran virus-Faisalabad[Pakistan: Faisalabad 1] (Accession number: AJ496286), Cotton leaf curl Kokhran virus-[Pakistan:Sakrand:2005] (Accession number: FN552006) , Cotton leaf curl Bangalore virus-[India:Bangalore:2004] (Accession number: AY705380) and C-59 partial sequence cloned.

4. CONCLUSION

Cotton leaf curl disease (CLCuD) has been a main focal point for begomo- virologists. The disease emerged as a local nuisance in Nigeria and was ultimately reported from Sudan with a reduction in cotton production in Gezira (Bailey, 1934). In India, it was reported from Sriganganagar, Rajasthan in 1993 (Ajmera, 1994). Pakistan being the largest exporter of raw cotton depends on its export for foreign exchange. To date, the decline in cotton production in the country is largely due to this disease. Emergence of the disease was reported from Multan in 1967 (Hussain et al., 2001). Since then it has been a major limitation to cotton production in Pakistan. The frequent mutations and recombination in the genomes of begomoviruses rendering them dominant to the disease resistance imparts special emphasis on the molecular characterization of the recombining viruses.

The agent underlying the cotton leaf curl disease (CLCuD) was not clearly characterized at the time of the disease incidence. Mullis et al., 1986 described polymerase chain reaction as an in vitro DNA amplification technique that utilizes various enzymes to amplify a DNA fragment. The technique led to molecular cloning of the amplified DNA. Partial as well as full length sequences of begomoviruses have thus been analyzed in detail. Since 1998, four evident begomovirus species causing CLCuD were prevalent in Pakistan but the begomoviral genome components were not recognized (Zhou et al., 1998). Briddon et al., 1999 reported the presence of DNA A associated with the disease and at the same time Mansoor et al., 1999 reported the presence of betasatellite along with DNA A.

Mixed infections were reported by Zhou et al., 1998 in cotton plants. The Several begomoviruses prevalent at that time and known to cause the disease infected the cotton host plants simultaneously. This had resulted in mixed infections. The resultant of these infections was the emergence of Cotton leaf curl Burewala virus (CLCuBuV) prevalent as a single dominant species infecting cotton in Pakistan. The virus has emerged as a serious threat to cotton production in Pakistan since 2001. It is a recombinant of Cotton leaf curl Kokhran virus (CLCuKV) and Cotton leaf curl Multan virus (CLCuMV). The virus has been previously reported form Vehari, Khanewal, Muzaffargarh, Layyah and Faisalabad. The recombination between the two has resulted in the loss of an intact transcriptional activator protein (TrAP). The coat protein of the CLCuBuV is contributed by the partial sequence of CLCuKV (Hina et al., 2012).

The question arises that why the C-59 partial sequence shares 99% sequence identity to the Cotton leaf curl Burewala virus (CLCuBuV) and Cotton leaf curl Kokhran virus (CLCuKV) partial sequences although both begomoviruses are distinct but closely related? The answer to the question lies in the fact that the virion sense sequences of the CLCuBuV are contributed by CLCuKV ((Amrao et al., 2010a).

The sequence on the virion sense strand of Cotton leaf curl Burewala virus (CLCuBuV) relates to the coat protein (CP) or V1 and the pre-coat protein or V2 (Glick et al., 2008). The universal primer CLCV1/2 used in the present research amplifies some part of CP and V2 along replication enhancer protein (REn) and some part of transcriptional activator protein (TrAP). Sequence analysis using open reading frame finder (ORF finder) gave a significant picture that the partial CP ORF present in the C-59 partial sequence is contributed by Cotton leaf curl Kokhran virus (CLCuKV) whereas partial REn ORF is related to CLCuBuV. Another ORF has been analyzed in Burewala strain. The ORF has been termed as C5. It has been shown to overlap with the CP ORF. The C5 present in the cloned C-59 partial sequence is related to CLCuBuV.

Since, CLCuBuV is a recombinant of CLCuKV and Cotton leaf curl Multan virus and the coat protein region of CLCuBuV was derived from CLCuKV that is most probable reason that the available sequence showed identity with CLCuBuV as well as CLCuKV. A complete characterization of full length virus will determine whether isolate C-59 is CLCuBuV or CLCuKV. Literature indicates that there is no existence of CLCuKV within the region and CLCuBuV is dominating within Indo-Pak region.

5. ACKNOWLEDGMENTS

The manuscript represents original and exclusive work generated in assistantship with Dr. Muhammad Tahir's HEC funded grant: Development and validation of transgenic resistance in Nicotiana benthamiana by the expression of artificial microRNA (amiRNA) carrying begomovirus. Thanks to Dr. Muhammad Tahir for his supervision and diligent guidance in order to reproduce this manuscript.

ABBREVIATIONS

µ	Micron
$(NH_4)_2SO_4$	Ammonium sulphate
AEV	*Ageratum enation virus*
AYVV	*Ageratum yellow vein virus*
CLCuAV	*Cotton leaf curl Alabad virus*
CLCuBuV	*Cotton leaf curl Burewala virus*
CLCuD	Cotton leaf curl disease
CLCuGB	*Cotton leaf curl Gezira Betasatellite*
CLCuGV	*Cotton leaf curl Gezira virus*
CLCuKV	*Cotton leaf curl Kokhran virus*
CLCuMB	*Cotton leaf curl Multan Betasatellite*
CLCuMV	*Cotton leaf curl Multan virus*
CLCuRV	*Cotton leaf curl Rajasthan virus*
CLCuSV	*Cotton leaf curl Shadadpur virus*
CP/V1	coat protein
CR	common region
CTAB	Cetyl trimethyl ammonium bromide
EDTA	Ethylene diamine tetra acetate
IR	Intergenic region
$MgCl_2$	Magnesium chloride
MP	Movement protein
NaOH	Sodium Hydroxide
NSP	Nuclear shuttle protein
OkEV	*Okra enation virus*
PaLCuV	*Papaya leaf curl virus*
REn/C3	Replication enhancer protein
Rep	Replication associated protein
SCR	Satellite conserved region
TrAp/C3	Transcription activator protein

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RANKL Signaling Pathway: A Potential Target for Antiresorptive Therapy in Rheumatoid Arthritis.

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic polyarthritic autoimmune condition characterized by severe bone erosion. Osteoclasts, the bone resorbing cells, are overly produced from the synovial inflammatory tissues in RA leading to excessive bone loss. RANKL-signaling pathway has been established to be the major pathway involved in osteoclastogenesis. This review highlights the role of around 40 osteoclastogenic factors involved in RANKL signaling and their potential to be targeted for antiresorptive therapy in RA. Furthermore, inhibitors of these proteins, which are already known to exhibit antiresorptive potential, have been reviewed. Elucidating new potential candidate therapeutic targets in the osteoclastogenesis pathway will open new avenues into the treatment and diagnosis of the arthritic conditions.

Keywords: RANK-RANKL signaling pathway, osteoclastogenesis, Rheumatoid arthritis, bone resorption

1. INTRODUCTION

Rheumatoid arthritis (RA), affecting about 1% of the world population, is a chronic polyarthritic autoimmune condition characterized by colossal joint destruction as a consequence of synovial hyperplasia (McInnes et al., 2011, Baron et al., 1989, Harris et al., 1990). Research studies of rheumatoid synoviocytes conducted to gain insights into the pathogenesis of bone erosion have led to the unraveling of their altered phenotype in RA patients (Davis et al., 2003). Bone and articular cartilage are invaded by pannus which is a fibrovascular structure formed as a result of proliferation of synoviocytes(Fuchs et al., 1989). Radiographic exams reveal that the symptoms of joint erosion in RA arise in very early stages and exacerbate with the advancement of the disease causing acute structural and functional impairment (Fuchs et al., 1989, van der Heijde et al., 1992). On that account, obstructing the process of bone erosion in RA becomes the most challenging and indispensible target to achieve. Extensive research indicates that ostoeclasts are the primary cells involved in the joint destruction in RA.

Osteoclastogenesis takes place at a momentously accelerated rate in Rheumatoid arthritis at a site at exterior to the marrow cavity, thereby leading to bone loss implicated in RA (Bromley et al., 1984). Consequently, abnormal bone resorption is the major pathology resulting in the functional impairment caused by rheumatoid arthritis (RA)(Bromley et al., 1984). It is supported by a multitude of research findings which suggest that synovial inflammatory tissue acts as a critical source of osteoclasts in RA by inducing localized osteoclastogenesis leading to loss in the bone mass (Fujikawa et al., 1996, Kotake et al., 1996, Takayanagi et al., 1997, Gravallese et al., 1998). Hence, overproduction of osteoclasts in RA suggests a significant connection between joint inflammation and structural damage (Schett et al., 2007). Identifying the molecular potential therapeutic targets in the osteoclastogenesis will open new avenues into the treatment and diagnosis of the arthritic conditions like RA.

Bone biology field has been greatly revolutionized since the unraveling of RANK-RANKL signaling pathway as the main pathway involved in osteoclastogenesis (Shigeyama et al., 2000). In this review, we seek to review various osteoclastogenic factors involved in RANKL signaling which have an implicated role in bone erosion associated with RA. We review therapeutic potential of thefactorswhich can be targeted to reduce bone resorption in RA.Moreover, we discuss about known inhibitors for these proteins which exhibit antiresorptive potential. Osteoclastogenic factors belonging to RANKL signaling pathway which have been reported in rheumatoid arthritis have been listed in the table. RANKL signaling pathway and osteoclastogenic factors involved in it have been demonstrated in the figure.

1.1 Bone Homeostasis

Bones are rigid yet dynamic endoskeletal organs which undergo remodeling throughout one's life span without any alterations in the size or shape (Teitelbaum et al., 2000). Maintaining normal mass in adult skeleton requires précised poise between the bone formation and bone degradation by specific types of cells (Zvi et al., 2007). Any change in the two processes leads to alterations in bone mass leading to bone-related disorders (Shcett et al., 2007, Soysa et al., 2012, Sturge et al., 2011).

1.1.1. Osteoclasts and the Bone Resorption

Osteoclasts are highly specialized, giant, unique bone resorbing polykaryons which originate from hematopoietic stem cells (Vignery et al., 2005). Myeloid progenitors undergo differentiation to form activated, bone resorbing osteoclasts via a process entailing the fusion of up to 20 mononuclear precursor cells, also known as syncytium (Rachner et al., 2011). Bone resorbing mechanism of

osteoclasts is regulated by two fundamental instrumentaries (Teitelbaumet al., 2000). First, special enzymes responsible for bone matrix degradation, such as cathepsins and matrix metalloproteinases, which cause cleavage of matrix molecules like collagen type-I and consequent removal of the non-mineralized substances from the bone. Second, vacuolar ATPase, a proton/protein pump giving rise to acidic environs between part of the osteoclast plasma membrane which demonstates metabolic activity, the disheveled boundary and the bone surface. This acidification results in solubilization of calcium from bone matrix by the cells (Li et al., 1996, Sundquist et al., 1990, Van Hille et al., 1995, Väänänen et al., 1990, Väänänen and Horton, 1995). The two aforementioned particularities allow osteoclasts to create a resorption pit by invading the bone. This resorption cavity can later be filled up by bone forming cells, osteoblasts. Osteoclasts are only found in close proximity of bones because the mineralized tissue provides them pivotal signals for their differentiation (Schett et al., 2007). Osteoclastogenesis and bone remodeling , therefore, normally occur within the bone and bone marrow (BM), except in certain pathological bone diseases, such as rheumatoid arthritis.

1.2. Role of RANKL Signaling Pathway in Bone Erosion Associated with RA

1.2.1. RANK-RANKL

RANK (Receptor activator NF-κB) and RANKL (Receptor activator of NF-κB Ligand) are the two major components of RANKL/RANK signaling pathway. Osteoblasts express RANKL protein as a type II transmembrane proteinin a soluble form resulting from the proteolytic cleavage (Wong et al., 1997, Anderson et al., 1997). Series of research studies on mice demonstrate that RANKL is indispensible in osteoclastogenesis (Theillet al., 2002). RANKL is subsequently demonstrated to bind to its receptor RANK. RANK, a type I TNFR-related transmembrane signaling protein, constituting 2-intracellular domains, expressed on the surface of osteoclasts, is activated upon RANKL binding (Woo et al., 2002). This binding results in the differentiation of osteoclast progenitor cells into mature osteoclasts followed by their activation (Lacey et al., 2000). In addition to the maintenance of survival of the mature osteoclasts, RANKL is responsible for the induction of cytoskeletal rearrangements thereby promoting bone resorption (Burgess et al., 1999, Kong et al., 1999). Moreover, osteoclast specific genes like cathepsin K and TRAF6 are also expressed as a consequence of induction by RANKL (Takahashi et al., 2011). A study entailing the immunohistological examination of tissue biopsies from patients of RA, OA and normal subjects showed the higher levels of RANKL in RA than in patients with OA or normal subjects (Zuoning Han et al., 1998).Denusumab, a human monoclonal antibody and a potent RANKL inhibitor, has been demonstrated to alleviate osteoclast resorption thus improving the symptoms of bone erosion (Kopper, 2012). Similarly, a RANK receptor inhibitor (RRI) peptide which targets a newly identified cytoplasmic motif of RANK protein, which has been reported to have a specific involvement in OC differentiation, has also shown to exhibit antiresorptive effects (Kim et al. 2009). Hence RANKL/RANK system is a potential target for pharmacological intervention in various bone disorders characterized with bone loss.

1.2.2. TRAF6

TRAF6 belongs to Tumor Necrosis Factor Receptor superfamily which is well-known for its role in osteoclastogenesis.Since RANKL lacks any intrinsic enzymatic activity, it recruits multifunctional second messangers like TRAF family members for signal transduction (Galibert et al., 1998, Wong et al., 1999, Wong et al., 1999, Lomaga et al., 1999). TRAF6 specifically induces NF-κB activity by inducing IKK complex either via TAK1 or aPKC-mediated phosphorylation (Walsh et al., 2008). In the classical NF-κB pathway, it binds to form a complex with p62 which interacts with aPKC which causes the phosphorylation of IKK-β, the critical regulator in osteoclastogenesis and bone loss (Duran et al., 2004, Sanz et al., 2000, Sanz et al., 1999). In the alternative NF-κB pathway, however, TRAF6 forms complex with TAK1 (TGFβ-activated kinase 1) and TAB1 and TAB2 adapter proteins. Upon stimulation, TAK1 phosphorylates NIK which activates IKK resulting in NF-κB activation (Mizukami et al., 2002). JNK pathway, apart from its activation by TAK1, is also activated by recruitment of p38 and its binding to TRAF6 complex by TAB1 thereby activating it (Lee et al., 2002, Ge et al., 2002). TRAF6 has been established to be the fundamental signaling adapter molecule since the TRAF6 knockout mice phenotype resulting from two independent studies show severe osteopetrotic symptoms (Naito et al., 1999, Takayanagi et al., 2000). TRAF-6 has been reported to be up-regulated in response to elevated levels of TNFα in RA patients (Aikawa et al., 2008).Moreover, inhibition of TRAF6 by IFNγ leads to a significant reduction in osteoclastogenesis which makes it a potential candidate for drug targeting (Anderson et al., 1997, Takayanagi et al., 2000). Another decoy peptide, T6DP, specifically targeting and inhibiting TRAF6 was also shown to prevent RANKL-TRAF6 mediated osteoclastogenesis (Poblenz et al., 2007). This implies that smallmolecular inhibition of TRAF6 can also be considered for antiresorptive therapy.

1.2.3. NF-κB

Rel or NF-κB, a transcription factor complex, plays significant role in numerous biological processes and hence implicated in a number of disorders including autoimmune disorders. It is activated by RANK and activates various downstream proteins involved in osteoclastogenesis. Activation of NF-kB is an immediate molecular event that takes place via recruitment of TRAF6 by RANK in osteoclastogenesis (Franzoso et al., 1997). Among five members of the NF-kB family, p50 (NF-κB 1) and p52 (NF-κB2) together play a role in osteoclastogenesis. Phenotype of p50-/- or p52-/- mice shows no bone related abnormally. However knockout mice for both of the genes show osteopetrotic phenotype implicating the essential role of the two genes in osteoclastogenesis (Iotsova et al., 1997, Takayanagi et al., 2002). NF-κB expression levels and its DNA binding activity have been demonstrated to be augmented in RA patients (Jeehee et al., 2002). Studies show that administration of an NF-κB inhibitor results in profound

reduction in osteoclastogenesis during the initial stages of the process as compared to the later stages thereby explaining its implicated role in the activation of early-stage immediate genes downstream of RANKL (Ishida et al., 2002). An inhibitor of NF-kB, has been shown to ameliorate arthritic symptoms via down-regulation of the key regulator of osteoclastogenesis in mice with collagen induced arthritis without impinging on any upstream molecules like M-CSF and RANK (Yin-Ji et al., 2012). Similarly, a peptide, known as NBD, has been shown to selectively inhibit NF- kB with significant reduction in osteoclast mediated-bone loss (Soysa et al., 2009).

1.2.4. Calcineurin-NFATc1

Nuclear factor of activated T-cells, cytoplasmic 1, belongs to NFAT family of transcription factors, which are involved in immune response. NFATc1 is the master regulator of the osteoclastogenesis owing to its indispensable role in the differentiation of osteoclasts (Takayanagi et al., 2002, Li et al., 2004, Kubota et al., 2007). Activation of NFATc1 is mediated by calcineurin which is a specific calcium-dependent serine-threonine phosphatase which dephosphorylates NFATc1 leading to its translocation into the nucleus. Inhibition by calcineurin inhibitors like cyclosporine A and FK506 results in major blockage of osteoclastogenesisthereby relieving pain in Rheumatoid arthritis rat models (Takayanagi et al., 2005,Magari et al., 2003; de la Pompa et al., 1998). Phenotype of mice deficient in NFATc1 exhibit acute osteopetrotic symptoms (Sitara and Aliprantis, 2010). In another study, in vitro experiments on NFATc1-/- demonstrate no differentiation of stem cells precursors into osteoclasts whereas in vivo analysis of NFATc1 knockout show fatal results in the embryo implicating the crucial role of NFATc1(Rangeret al., 1998, Houet al., 2001). Phenotype of TRAF6 knockout mice exhibits impaired induction of NFATc1 implying that it is one of the major immediate targets of NF-kB during the initial stages of osteoclastogenesis (Li et al., 2004). It is corroborated by the studies which show that P50/p52-knockout mice show no induction of NFATc1 (Takatsuna et al., 2005). Expression profiling of RA patients shows augmented levels of NFATc1 (Li et al., 2012). Inhibiting effects of various compounds on NFATc1 activity have been studied including cinnamaldehyde, NGDA and obovatol (Kim et al., 2013, Tsuji-Naito et al., 2008, Yamamoto et al., 1992).

1.2.5. p62

P62 is a scaffold, adaptor, ubiquitin binding- protein encoded by SQSTM1 which modulates several critical cell functions like protein turnover, internalization of receptors in cell signaling pathways, transcription of genes as well as modulation of protein-protein interactions (Moscatet al., 2007). Besides these, its significant role has been demonstrated in TRAF6 mediatedspecific activation ofNF-kB (Geethaet al., 2002). Mutations have been observed in SQSTM1 in Paget's bone disease which is characterized by high osteoclastogenic activity and thus excessive bone resorption (Morissetteet al., 2006). P392L mutation in p62 in PBD has been shown to upregulate NFATc1, the key regulator of osteoclastogenesis (Duran, et al., 2004, Sundaramet al., 2011). Phenotype of cells transfected with this mutation exhibit augmented bone erosion (Kuriharaet al., 2007) Furthermore, phenotype of SQSTM1 knockout mice results in no activation of IKK or NF-kB, no NFATc1 production and hence no osteoclastogenesisthus conspicuously playing a crucial role in osteoclastogenesis (Duran et al, 2004). Levels of p62 also need to be elucidated in other bone-related disorders characterized by increased osteoclastogenesis like osteoarthritis and rheumatoid arthritis. Not only that p62 can be targeted for therapeutic intervention, but since it works in complex with TRAF6, competitive inhibitors for TRAF6 can be designed and tested in order to reduce osteoclastogenesis by preventing the downstream activation of NF-kB pathway.

1.2.6. NIK

NF-kB -inducing kinase (NIK) is a serine/threonine protein-kinase encoded by MAPKKK14 gene. Activated by RANKL, NIK is thecontrolling component of the alternative NF-kB pathway. It is activated in response to its phosphorylation by TAK1 which occurs via formation of a complex between TRAF6 and TGF beta-activatedkinase 1 (TAK1) and adaptor proteins TAB1 and TAB2. As a result, IKK complex is activated thereby inducing NF-kB pathway (Mizukami et al., 2002). Phenotype of NIK knockout mice exhibit resistance to RANKL-mediated osteoclastogenesis suggesting their role in bone resorption by OCs (Novacket al. 2003). NIK has been demonstrated to modulate osteoclast function in rheumatoid arthritis (Aya et al., 2005). It can hence be targeted for therapeutic intervention to treat arthritic symptoms by preventing bone destruction by OCs.

1.2.7. IKK Complex

IKK complex, responsible for NF-kB activation entails three subunits includingIKKα and IKKβ, and a regulatory subunit, IKKγ thereby contributing to the production of osteoclasts and thus the increased bone resorption in inflammatory arthritis (Rothwarf and Karin, 1999). In alternative NF-kB pathway, NF-kB activation is dependent on phosphorylation of IKKα by NIK (Senftleben, et al., 2001, Xiao, G et al., 2001) In classical NF-kb pathway, however, IKKα has been shown not to have a relevant role in osteoclastogenesis in contrast to IKKβ and IKKγ, where mice lacking IKKβ develop osteopetrotic sypmtoms (Ruocco et al., 2005). IKKβ has been shown to be the central role player in osteoclast formation and its constitutive activation can result in bone erosion (Otero et al., 2010). A multitude of small molecule inhibitors for IKK have been developed in tested in vitro and in vivo for their anti-osteoclastogenic activities. Some of these include BMS-345541 (BMS) and parthenolide (PAR) which act directly against IKKαand IKKβ respectively thus blocking their kinase activity (Hehner et al., 1999,Kwok et al., 2001, Yip et al., 2004, Burke et al., 2003, Yang et al., 2006). IKK complex thus has the potential to be targeted for therapeutic intervention for treatment of diseases involving bone erosion.

1.2.8. ITAM

In addition to RANKL, osteoclast differentiation also needs certain costimulatory signals produced byDAP12 and Fc receptor common γ (FcRγ) which belong to ITAM class of adaptors (Collin-Osdoby et al., 2001, Chow et al., 1992). The

concerted activity of ITAM and RANKL signaling leads to the activation of NTATc1, the key modulator of osteoclastogenesis (Negishi-Koga and Takayanagi, 2009). OSCAR is an osteoclast-specific, Ig-like, osteoclast associated receptor protein, expressed on pre-osteoclasts and is involved in the modulation of osteoclasts function and differentiation (Kim et al., 2005,Kim et al., 2002, Ohshima et al., 2002). It is induced by RANKL and is expressed in last phases of maturation of osteoclast precursors. OSCAR has been shown to particularly signal via FcRγ and therefore can rescue osteoclastogenesis even in the absence of DAP12. OSCAR's expression in regulated by MITF and PU.1 transciption factors (Donnenberget al., 2006). It has been reported to be expressed in monocytes of RA patients thereby regulating osteoclast differentiation and ultimately increased bone resorption (Hermanet al., 2008).Mice deficient in both DAP12 and FcRγ, however, show osteopetrotic symptoms in the absence thus implicating their critical role in bone resorption (Koga et al 2004, Mocsai et al, 2004, Paloneva J, 2003).Tyrosin residues within the ITAMs are phosphorylated by SKFs (Src family kinase, with c-src being the most dominant member being expressed in osteoclasts) which then bind to SyK leading to its activation (Pitcher and van Oers, 2003, Futterer et al., 1998, Brdicka et al., 2005).Src, involved in osteoclastogensis, is activated by RANKL. Mutations leading to deficiency in src leads to osteopetrotic symptoms thus implying its potential as a therapeutic target for RA (Kawaji et al., 1995).SyK is another tyrosin kinase which has been demonstrated to play a role in osteoclast function (Faccio et al., 2003; Mocsai et al., 2004).SyK further leads to the activation of other adaptors including BLNK and SLP-76 which cause the recruitment of RANK and PLCγ-activated Tec kinases to osteoclastogenic signaling complex thereby enabling maximal influx of calcium.Btk and Tec are the major kinases from the Tec kinase family specifically expressed in osteoclasts. Phonotype of doubly deficient mice for both of these exhibit osteopetrotic symptoms thus implicating their indispensible role in bone erosion, ITAMs are hence, candidates for therapeutic intervention (Shinohara et al., 2008).

1.2.9. PLCγ

PLCs are the family of enzymes involved in the regulation ofintracellular calcium levels and expression of NFAT. They breakdown phospholipids present in biomembranes into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) which activate PKC and directly augment the levels of calcium by liberating it from the endoplasmic reticulum repository respectively. PLCγ2 is a member of PLCγ family and is dependent on tyrosine residue phosphorylation for its enzymatic action. It has been demonstrated that PLCγ2 knockout mutation results in mice exhibit abnormally increased bone mass in vivo. In vitro, the osteoclast precursors result in no differentiation of OC precursors into mature osteoclasts, no NFATc1 expression , defective phosphorylation of JNK, c-Jun, and IκBα phosphorylation and ultimately impaired AP1 and NF-κB activation.Moreover, PLCγ inhibitor, U73122, has shown to prevent OC development in the culture of osteoclasts thus implicating its role in osteoclast differentiation. It has been reported that PLCγ2 undergoes complex formation with Gab2(Mao et al., 2006) Gab2 (grb-2-assocaiated binding) protein belongs to another family of adaptor molecules involved in receptor signaling which also connects RANK with various downstream pathways involved in osteoclastogenesis. This is evident from the study that Gab2 deficient mice exhibit osteopetrotic symptoms in vivo thus implicating defects in osteoclastogenesis. Thus Gab2 plays a momentous role in RANKL-mediated osteoclastogenesis and is a target for therapeutic intervention (Wada et al., 2005). The PLCγ2-Gab2 complex is essential for the phosphorylation of Gab2 and that is how it is recruited to RANK for osteoclastogenesis. So PLCγ2 modulates osteoclastogenesis via ITAMs as well as Gab2 and hence is a potential candidate for antiresorptive therapeutic intervention (Mao et al., 2006).

1.2.10. AP-1/c-fos

AP-1 is a transcription factor complex with c-Fos being an essential constituent of the AP-1 transcription factor complex activated via RANK induction (Wagner et al., 2005, Johnson et al., 1992, Teitelbaum et al., 2004). C-Fos belongs to the Fos family of transcription factors and is responsible for the induction of NFATc1, the master regulator of osteoclastogenesis. This makes c-Fosan indispensible factor for osteoclastogenesis. Complete blockage of osteoclastogenesis occurs as a result of c-Fos knockout mutations in mice thereby displaying severe osteopetrotic symptoms (Wang et al., 1992, Kim et al., 2013). Levels of AP-1 and c-fos have not only been reported to be higher in RA patients but the bone resorption process in RA is also implicated to be the ultimate result of c-fos overexpression (Zuoning et al., 1998, Kurokiet al., 1993, Kurokiet al., 1993, Kurokiet al., 1994, Miyauchi et al., 1994, Trabandt et al., 1992, Shiozawa et al., 1997). An inhibitor designed by pharmacophore modeling was shown to be able to selectively inhibit AP-1/c-fos thereby resolving the arthritic condition (Harris et al., 1990). This implies that function of these proteins is indispensible for osteoclastogenesis making them potential therapeutic targets (Takayanagiet al., 2005, Asagiriet al., 2005). An attempt to inhibit AP-1 expression at transcriptional level using a sequence specific AP-1 binding oligonucleotides in has already been shown to be successful in alleviating bone erosion symtoms in a dose-dependent, sequence specific manner (Aliprantiset al., 2008).A new drug, T-5224, has also been computationally designed to inhibit AP-1/c-fossuggesting their therapeutic potential (Miyazaki et al., 2012).

1.2.11. Cathepsin K

Cathepsin Kis a cysteine protease responsible for the destruction of protein constituents of bone matrix like osteonectin, and collagens types I and II produced by synovial fibroblasts and bone resorbing macrophages, (Goto et al., 2003). It is akey regulator of osteoclast function, Cathepsin K and therefore, has an implicated role in diseases characterized by accelerated rates of joint destruction like rheumatoid arthritis, osteoarthritis and osteoporosis (Riemanet al., 2001, Kim et al., 2005). Morever, Cathepsin K has been shown to be up-regulated in response to IL-1 which is a proinflammatory cytokine and a strong inflammatory marker of RA

(Kamolmatyakul et al., 2004).Its expression, like that of TRAP (Houet al., 2001, Matsumoto et al., 2004, Anusaksathien et al., 2001), calcitonin receptor (Houet al., 2001, Matsumoto et al., 2004, Anusaksathien et al., 2001, Goll et al., 2003) and β3 integrin genes (Matsuo et al., 2006), is also modulated by NFATc1 (Matsumoto et al., 2004, Crotti et al., 2006).Cathepsin K, levels in sera of RA patients are specifically elevated and it has been shown to be responsible for joint erosion via degradation of articular cartilage since it is localized at the sites of synovial bone erosion (Martin et al., 2005). Hence, Cathespsin K is not only a potential therapeutic target for anti-rheumatic drug intervention but may also prove to be a highly exclusive diagnostic marker for RA. A specific inhibitor of cathepsin K, Odanacatib, has recently been pharmacologically evaluated for the treatment of bone loss in osteoporosis (Stoch et al., 2013).

1.2.12. PU.1

PU.1, an ETS family transcription factor, exhibits favored expression in hematopoietic stem cells and plays a prodigious role in differentiation and formation of osteoclasts via direct interaction with MITF (Simonet al., 1998, Singh et al., 1999, Tondraviet al., 1997, Luchin et al., 2001). Phenotype of knockout mice for PU.1 exhibit osteopetrotic symptoms as a result of osteoclastogenic inhibition at initial stages of differentiation (Tondravi et al., 1997). FLS of RA patients also exhibit elevated levels of PU.1 transcription factor (Itoh and Nagatani, 2012). CC-4047, an immunomodulatory derivative of thalidomide has been shown to prevent osteoclastogeneis by imposing its inhibitory effects on PU.1 (Donnenberget al., 2006). This makes PU.1 a candidate for antiresorptive therapeutic intervention.

1.2.13. MITF

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor involved in osteoclast development as a result of its induction downstream of RANKL (Lu et al., 2010). NFATc1-dependent enhancement of osteoclast differentiation has a direct correlation with the elevated levels of MITF (van der Kraan et al., 2013). Moreover, phenotype of knockout mice exhibit osteopetrotic symtoms thereby implicating its significant role. Transcriptional regulator MITF results in the OC differentiation as a result of activation by p38. In collaboration with PU.1, expression of osteoclast specific genes like TRAP, cathpsin K and OSCAR is also modulated by MITF via M-CSF and RANKL signaling downstream pathways (Manskyet al., 2002, So et al., 2003; Hu et al., 2007). Levels of MITF have been shown to be reduced in response to Tacrolimus and Cyclosporin A in RA which corroborates its potential to be considered as a target for anti-rheumatic therapy (Miyazaki et al., 2007).

1.2.14. TRAP

TRAP (Tartrate-resistant acid phosphatase), a glycosylated monomeric metalloenzyme, is an established osteoclast specific marker which degrades the collagen by producing highly reactive ROS thereby leading to bone destruction (Halleenet al., 1999). TRAP 5b is a specific indicator of bone resorption and hence possesses great diagnostic importance (Halleen et al., 2000). Total TRAP activity has been demonstrated to be elevated in sera of patients with RA (Janckila et al., 2002). Moreover, phenotype of mice which have a knockout mutation for TRAP exhibit osteopetrotic symtoms thus showing their role in osteoclast function (Hayman et al., 1998). An inorganic poly(P) chain constituting 300 phosphate residues has been recently shown to strongly bind and block the activity of TRAP resulting in no formation of resorption pit required for bone degradation (Harada et al., 2013). TRAP is therefore a potential for osteoclast specific antiresorptive therapy for Rheumatoid arthritis.

1.2.15. Carbonic Anhydrase II

Carbonic anhydrase II belongs to carbonic anhydrase family of enzymes responsible for catalysis of carbon dioxide hydration in a reversible fashion. Bone resorption by osteoclasts requires a peculiar, poorly perfused, acidic, peripheral bone milieuwhich is achieved by the vacuolar proton pump activation (Vaes, 1988, Blair et al., 1989, Vaananen et al., 1990, Sundquist et al., 1990). CAII facilitates the proton production from H2O and CO2 thereby enabling the radical acidification of the resorption space (Sly et al., 1983). Distruption in CAII gene in vivo results in no bone resorption thereby implicating its critical role in optimal bone resorption process (Hall and Kenny, 1987; Raiszet al., 1988; Hott et al., 1989, Hall et al., 1991, Gay and Mueller, 1974). It has been shown to be selectively activated downstream of RANKL (Takayanagi et al., 2002). In mouse calvaria cultures, a specific inhibitor of CAII, acetazolamide, exhibits antiresorptive activity in a dose dependent, Ca2+ dependent pH-regulated manner (Lehenkari et al., 1998). Similar to acetazolamide, celecoxib and JTE-522 also inhibit osteoclast differentiation and activity which were tested in arthritic rat model for RA and showed reduced bone resorption (Katagiri et al., 2006). Levels of CAII in Rheumatoid arthritis need to be checked since it has the potential to be therapeutically targeted.

1.2.16. SH3BP2

SH3 (domain-binding protein 2), plays its role in increased osteoclastogenesis in a multitude of ways as it results in the elevated levels of both NFATc1 and the osteoclast specific gene, TRAP. Moreover, overexpression of SH3BP2 in RAW247.6 cells lead to the increased nuclear translocation of NFATc1 and hence increased osteoclastogenesis (Steven et al., 2008). Its function is critical for the calcium influx (Chenet al., 2007). It has been demonstrated to have over abundant levels in autoimmune arthritis thereby contributing to bone erosion (Gallanti et al., 2014). It is therefore a potential target for pharmacological intervention.

1.2.17. β3 Integrin

Integrins are the heterodimer proteins which are responsible for the cell-matrix interaction. v 3, a receptor for vitronectin, which is an extracellular matrix glycoprotein, has been suggested to be of paramount importance in bone resorption as it has been found to be expressed in osteoclasts in large amounts and has been implicated to modulate the attachment of sealing zone to the bone matrix (Reinholt et al.,

1990, Nakamura et al., 1996, Holt et al., 1998). Mice lacking v 3 have been shown to exhibit osteosclerotic symptoms suggesting their role in bone mass regulation (Kevin et al., 2000). In rheumatoid arthritis, not only integrins but also their ligands like collagen, fibronectin and their degradation products have been shown to have augmented levels (Postigo et al., 1993; Lowin and Straub, 2011). Antibodies against v 3 and peptides having RGD, like kistrin and echistatin, exhibit inhibitory effects on bone resorption activity (Horton et al., 1991, Lakkakorpi et al., 1991, Fisher et al., 1993). Furthermore, two inhibitors of v 3, SC-56631 and SC-65811 have also evaluated for their antiresorptive activity and hence elucidate its potential as a target for pharmacological intervention (Carron et al., 2000).

1.2.18. CaMKIV-CREB

CaMKIVis a Ca2+/calmodulin-dependent protein kinase family. CaMKIV-CREB pathway has been reported to be crucial for the differentiation and function of osteoclasts by induction of NFATc1. Differentiation of osteoclast precurors into mature osteolclasts has been demonstrated to be abrogated by blockade by CaMK inhibitor, KN-93 as well as in CREB knockout experiments (Kojiri et al., 2006). Moreover, CaMKIV gene knockout experiments and inhibition by pharmacological agents result in decreased phosphorylation of CREB, a cellular transcription factor and hence the decrease in levels of c-fos which has a major role in the induction of NFATc1. This provides basic for new therapeutic strategies (Sato et al., 2006).

1.2.19. MAPK Cascade

In addition to NF-kB/c-fos pathway, binding of RANK to its receptor RANK also leads to the subsequent activation of MAPK pathway including extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Li et al., 2011). MAPK signaling in RA occurs via a three-level cascade of kinases called MAPKs. MAPKs or MAP kinases are the serine/threonine/tyrosine-specific protein kinaseswhich belong to (CDK/MAPK/GSK3/CLK) group of kinases. Involvement of MAPK cascade has been reported in rheumatic conditions (Sweeney et al., 2004; Schett et al., 2000). MAPKKK or MEKKs are the most upstream kinases which work in connection with GTPases like Rho and Ras, the latter being associated with chronic synovial inflammation (Marinissen et al., 2001).Nitrogen-containing biphosphate drugs inhibit Ras thereby showing anti resorptive effects (Luckman et al., 1998; Fisher et al., 1999). MEKKs activate downstream MAPKKs which in turn, activate p38 and JNK pathways. MKK-7 specifically activates JNK pathway while MKK-4, in some cases also lead to phosphorylated activation of p38MAPK in addition to JNK (Yan et al., 1994, Blank et al., 1996, Gerwins et al., 1997).MEKK-1 has been shown to have elevated levels in RA patients as well as in cultured synoviocytes, which also overly express MEKK-2 (Hammaker et al., 2004). Other MAPKKKs like TAK1 and MTK specifically activate p38MPAK via induction of MKK-3 and MKK-6 (Takekawa et al., 1997, Yamaguchi et al. 1995).TAK1 has been reported to have augmented levels in synovial tissues and synoviocytesin RA patients (Hammaker et al., 2004). MKK-3 and MMK-6 have been reported to specifically induce p38 whereas MEK-1 and MEK-2 are basically responsible for the activation of ERKs (Boyle et al., 2014; Fanger et al., 1997). An inhibitor of MEK-1 has shown to block differentiation of osteoclasts precursors thus implicating its role in osteoclastogenesis. Blocking of Mek ½ via a strong specific inhibitor ARRY-162 in AIA, which has entered phase-II clinical trials corroborates their therapeutic potential (Lindstrom et al., 2010). Hence, pharmacological intervention which blocks their activity or JNK knockout transfection results in repression of RANKL-mediated osteoclast formation (Ikedaet al., 2004).

MKKs, downstream to MAPKKKs, are responsible for phosphorylating p38MAPK. The most significant MKKs are MKK-3 and MKK-6 which is evident from the p38 lacking phenotype of the mice with double knockout mutation for both of these MKKs (Cong et al., 1999). MKK-3 leads to the preferential induction of p38MAPK in synovial fibroblast as a result of their exposure to TNF and IL1 (the proinflammatory cytokines) (Inoue et al., 2006, Moriguchi et al., 1996). Overexpression of p38 also leads to the increased expression of MKK-3 and MKK-6 in the patients with RA (Chabaud-Riou et al., 2004). As a result, p38 is strongly expressed in synovial membrane and in the osteoclasts at the site of synovial invasion (Hayer et al., 2005). P38 and other MAPKs also control regulation of MMPs.

Hence increased MAPK levels implicate increased MMP and thus increased collagen destruction(Liacini et al., 2003, Suzuki et al., 2000). This is corroborated by alleviation of cartilage destructive symptoms via p38 blockade (Zwerina et al., 2006) In addition; SCIO-469 another p38 inhibitor has shown to block osteoclastogenic process by preventing formation of osteoclasts (Nguyen et al., 2006). A JNK-specific inhibitor, SP600125, has also shown to inhibit anti-apoptotic characteristics of osteoclasts (Ikeda et al., 2008). Hence, the accumulating evidence suggests the therapeutic potential of MAPK cascade.

1.2.20. PPARα

PPARα, is a member of nuclear receptor family, is a transcription factor activated by ligand.There exist three subtypes of PPAR namely PPAR α β, and γ and all of them have been shown to be expressed in osteoclasts (Cernuda-Morollon et al., 2002; Mano et al., 2000; Mbalaviele et al., 2000). The levels of PPAR-α have been demonstrated to be elevated in the synovial fluid of patients with rheumatoid arthritis. A small ligand, fenofibrate designed to inhibit PPAR-α has shown to result in reduced bone erosion making it a potential target for anti-rheumatic therapy (Okamoto et al., 2005).

2. CONCLUSION

Bone disorders like rheumatoid arthritis are characterized by bone erosion which is a consequence of abnormal regulation of balance between bone formation and bone resorption. Osteoblasts and osteoclasts are responsible for maintaining proper bone mass. Since there has been accumulating evidence suggesting the increase in osteoclastogenesis in RA, joint destruction is considered as the major pathology associated with RA. Although exact etiology of RA remains

obscure, several genetic factors have been found to be associated with osteoclastogenesis which play their role in differentiation, formation, activation, cell-cell fusion and survival of osteoclasts thereby enabling excessive bone erosion. These mainly constitute the set of genes playing their roles in RANKL signaling pathway including NFATc1, the master regulator of osteoclastogenesis. RANKL signaling pathway activates multiple downstream pathways including MAPKs (p38, ERK and JNK), classical and alternative NF-κB pathways and CamKIV-CREB all of which eventually result in the AP-1/c-fos/NFATc1 induction leading to stimulation of osteoclastogenic pathway. Expression profiles of the patients with bone related disorders like osteoporosis, osteoarthritis and rheumatoid arthritis have shown to exhibit augmented levels of these osteoclastogenic factors and hence the excessive bone degradation. Natural and synthetic inhibitors against some of these factors have already been evaluated from pharmacological standpoint and some of them have shown their anti-arthritic activity by blocking osteoclastogenesis. For example,targeting NFATc1 by inhibitors like obovatol and cinnamaldehyde inRA has shown antiresorptive effects. Hence, using derivatives of already used compounds can therefore help elucidate more potent actives. In addition, expression levels of certain proteins activated downstream of RANKL signaling pathway like carbonic anhydrase II need to be elucidated in Rheumatoid arthritis and osteoarthritis and can be targeted for pharmacological intervention.

Table 1. List of osteoclastogenic factors involved in RANKL signaling reported in RA and their inhibitors

Protein	Family	Inhibitors	References
Ras	GTPase	N-BP drugs	Marinissen et al., 2001 Luckman et al., 1998 Fisher et al., 1999
MEK 1/2	MAPKK	ARRY-162	Wright et al. Lindstrom et al., 2010
TAK1	serine/threonine protein kinase family	_____	Hammaker et al., 2004
P38	MAPK	SCIO-469	Hayer et al., 2005 Nguyen et al., 2006 Inoue et al., 2006 Moriguchi et al., 1996
JNK	MAPK	SP600125	Ikeda et al., 2008
MKK-3	MAPKK	_____	Chabaud-Riou et al., 2004 Cong et al., 1999
PPARα	nuclear receptor family	Fenofibrate	Okamoto et al., 2005
MKK-6	MAPKK	_____	Chabaud-Riou et al., 2004 Cong et al., 1999
αvβ3	Integrins	Kistrinechistatin SC-5663 SC-65811	Postigo et al., 1993 Lowin and Straub, 2011 Carron et al., 2000
TRAP	glycosylated monomeric metalloenzyme	inorganic poly(P) chain	Harada et al., 2013
MITF	helix-loop-helix leucine zipper transcription factor	TacrolimusCyclosporin A	Miyazaki et al., 2007
PU.1	ETS family transcription factor	CC-4047	Itoh and Nagatani, 2012 Donnenberget al., 2006
Cathepsin K	cysteine protease	Odanacatib	Martin et al., 2005 Stoch et al., 2013

AP-1/c-Fos	Transcription factor	AP-1 binding oligonucleotides T-5224	Zuoning et al., 1998 Kurokiet al., 1993 Kurokiet al., 1994, Miyauchi et al., 1994, Trabandt et al., 1992, Shiozawa et al., 1997 Aliprantiset al., 2008 Miyazaki et al., 2012
OSCAR	osteoclast-associated receptor	_____	Hermanet al., 2008
NIK	serine/threonine protein-kinase	_____	Aya et al., 2005
Calcineurin	calcium-dependent serine-threonine phosphatase	cyclosporine A FK506	Takayanagi et al., 2005 Magari et al., 2003 de la Pompa et al., 1998
IKK complex		BMS-345541 (IKKα) parthenolide (IKKβ)	Rothwarf and Karin, 1999 Hehner et al., 1999 Kwok et al., 2001 Yip et al., 2004 Burke et al., 2003 Yang et al., 2006
TRAF6	Tumor Necrosis Factor Receptor superfamily	IFNγ T6DP	Aikawa et al., 2008 Anderson et al., 1997 Takayanagi et al., 2000 Poblenz et al., 2007
RANK	*type I TNFR-related transmembrane signaling protein*	*RANK receptor inhibitor (RRI) peptide*	*Kim et al. 2009*
RANKL	*type II transmembrane protein*	*Denusumab*	*Zuoning Han et al., 1998* *Kopper, 2012*
Carbonic anhydrase II	*Carbonic anhydrase*	*acetazolamidecelecoxib* *JTE-522*	*Lehenkari et al., 1998* *Katagiri et al., 2006*
SH3BP2	*SH3 domain-binding protein*	_____	*Steven et al., 2008* *Gallanti et al., 2014*

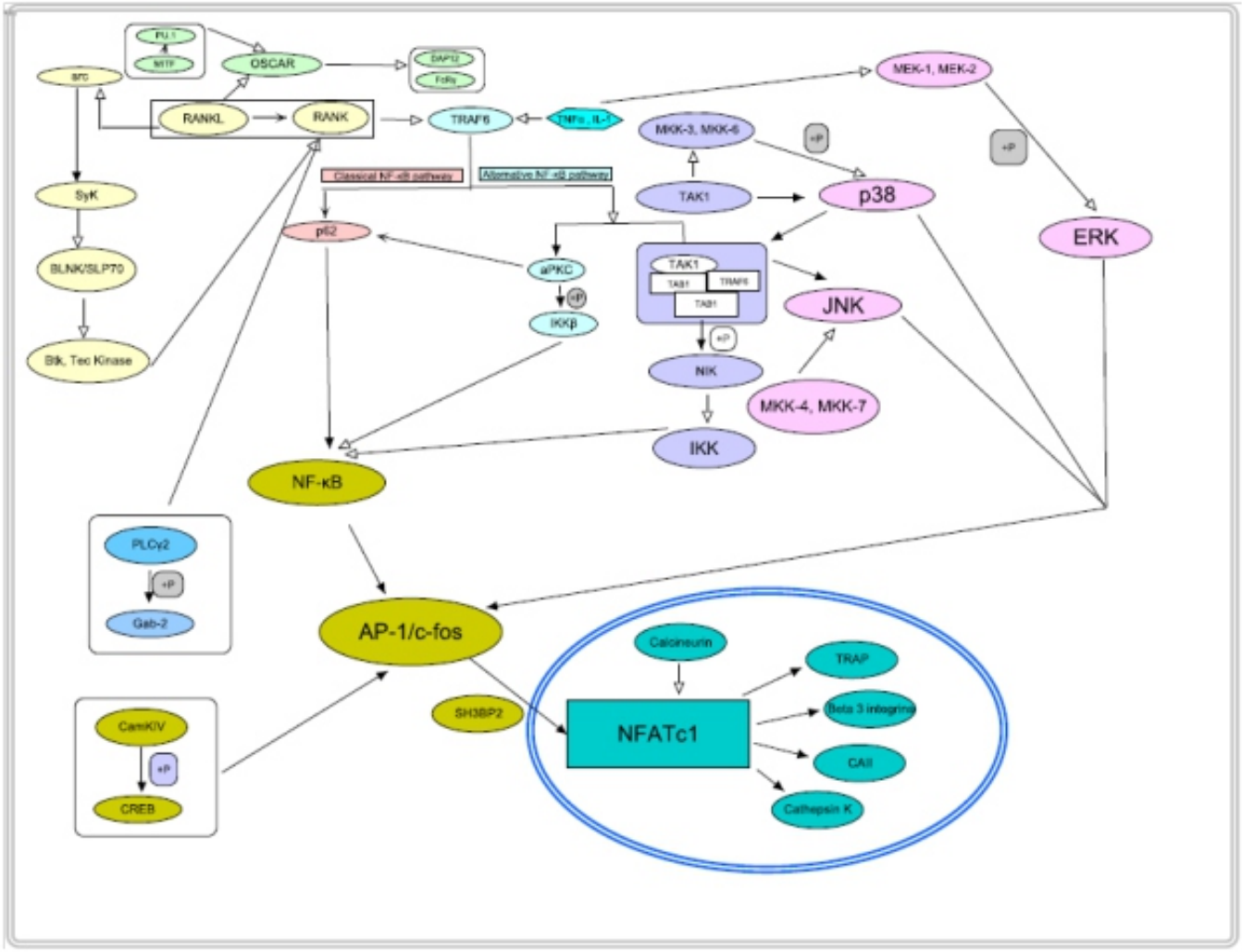

Figure 1. Osteoclastogenic Factors in RANKL Pathway

Binding of RANKL to its receptor RANK leads to the activation of TRAF6 thereby inducing downstream classicial and alternative NF-κB pathways. Classical pathway is induced via induction of p62 by aPKC. The alternative NF-κB pathway, is activated either via phosphorylation of IKKβ by aPKC or by phosphorylation of IKK complex by NIK as a result of TRAF6/TAK1/TAB1/TAB2 complex formation. This also results in the activation of MAPKs like p38, ERK and JNK via MEKKs and MEKs which induce AP-1/c-fos. In addition to its activation by NF-kB, AP-1/c-fos is also activated by CREB after its phosphorylation by CamKIV. AP-1/c-fos induction leads to the activation of NFATc1 while SH3BP2 plays its role in its nuclear translocation. Activation of NFATc1 leads to the downstream expression of various osteoclast specific genes like Cathepsin K, carbonic anhydrase II, TRAP and β3 integrins. RANKL also induces downstream activation of src and OSCAR which lead to activation of Tec Kinases and ITAMs like DAP12 and FcRγ respectively, all essential for osteoclastogenesis. PLCγ2 forming complex with gab2 thereby phosphorylating it leads to the recruitment to RANK to its osteoclastogenic signaling complex.

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Signaling Pathways Involved in Rheumatoid Arthritis: Targets for New Therapeutic Interventions

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ABSTRACT

Rheumatoid arthritis is categorized as a systematic autoimmune disease which causes chronic disabilities exclusively in bones that are aligned with synovium. RA aetiology is still unknown but previous studies have coined that several number of factors play a significant role e.g. environmental and genetic factors. Cellular signalling pathways orchestrate the inflammatory response that regulates various cellular functions like cellular progression, proliferation, death and secretion of signalling molecules (pro and anti-inflammatory cytokines) in response to genetic and environmental stimuli. These regulatory pathways are tightly controlled and naturally activated by ligands that attach to their respective receptors on the cell surface. In diseased state, these signalling pathways escape the normal control mechanisms, resulting in intensification of cytokines and chemokines, transcription factors and mediatory proteins that disrupt normal cell processes and might bring about auto-destructive consequences such as in the case of rheumatoid arthritis. The review highlights multiple levels of targeting molecules in signalling pathways that may be potential diagnostic markers and also attempts to underline potential therapeutic targets.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterized by inflammation of joints especially small joints of hands and feet ultimately leading to joints deformation and bone destruction. It involves imbalances in pro-inflammatory and anti-inflammatory cytokines levels (Paunovic & Harnett, 2013). RA develops as a result of combination of genetic and environmental factors. About 1% of the adults all over the world are reported to be affected by RA. Women are 2-3 times more prone to this disease as compared to men (Tobon, Youinou et al 2010) A well-defined twin study showed the involvement of genetic factors in RA accounting for 15-30% concordance rate in case of monozygotic twins and 5% in case of dizygotic twins (McInnes & Schett, 2011).

RA is best characterized by elevation in the levels of proinflammatory cytokines leading to persistent inflammation and subsequent damage to bone and cartilage. The signalling pathways of these inflammatory cytokines have emerged to have promising potential to be targeted by well-defined therapies thereby inhibiting inflammation and disease pathogenesis (Schett, 2011).

Depending on the progressive autoimmune nature, certain complexities are linked within a number of organ systems, which worsens the condition. The basis for such chronic inflammatory processes are sustained communication networks between cells within a tissue type and between different tissues. Signalling pathways lead to inflammation and various other cellular processes comprising of apoptosis, cell proliferation and cell differentiation. The signalling cascades when escape the balanced mechanisms, lead to disease pathogenesis or cellular destruction as observed in systemic rheumatoid arthritis (Tak, 2009).

This review highlights various signalling pathways involved in pathogenesis of RA e.g. MAPK pathway, Wnt signalling pathway, JAK/STAT pathway, NFκB signalling and TLR signalling, their activation, function, role in regulating cytokines and promoting inflammation and subsequent tissue destruction and also aims to summarize potential therapeutic targets identified so far in order to provide an efficient therapy for the disease.

1.1. MAPK Signalling in Rheumatoid Arthritis

Cellular responses are assimilated to environmental stresses by a group of signal transducing enzymes known as mitogen-activated protein kinases abbreviated as MAPKs(Inoue et al., 2006). MAPKs play important role in B / T Cell Receptor (BCR/TCR) signaling and also involved in signaling via other receptors such as toll-like receptors (TLRs), Interleukin Receptors including IL-1R, IL-17R and TNF-α. MAPKs are also regarded as important regulators involved in production of proinflammatory cytokines including TNF-α, IL-12, IL-23, IL-1, and IL-6 (Harnett et al.,2005; kolls and Linden, 2004; Lubberts et al.,2005). MAPKs consist of a highly conserved family of serine/threonine kinases involved in the regulation of important cellular processes including survival and cell growth, programmed cell death or apoptosis, proliferation/division, differentiation/specialization and inflammations in response to different stress molecules (Thalhamer et al., 2008). In mammals, three major types of MAPKs are reported namely, Extracellular signal Regulated Kinases (ERKs), C-Jun N-terminal kinase (JNK) and p38 MAPKs. JNK and p38 MAPKs collectively forms a family of kinases named as Stress Activated Protein Kinases (SAPKs)

(Thalhamer et al., 2008).

A signaling cascade, highly conserved among the species, leads to the activation of MAPKs (Dong et al., 2002; Johnson and Lapadat, 2002). First, MAPK Kinase Kinases (MAPKKK or MKKK) are activated that leads to activation of MAPK kinases (MAPKK or MKK) that ultimately leads to the activation of MAPKs in a complex signaling cascade (Figure 1A).MAPKKKs are serine/threonine protein kinases that activates MAPKKs through phosphorylation, while MAPKKs are protein kinases involved in activation of MAPKs through threonine and tyrosine phosphorylation in its highly conserved Threonine – X – Tyrosine motif (Thalhamer et al., 2008). The X residue in the motif is different for each of the MAPK class such as ERK contain Threonine - Glutamic acid – Tyrosine motif, Threonine – Proline -- Tyrosine motif in JNK while Threonine – Glycine – Tyrosine motif in p38 MAPKs. T–X–Y motif phosphorylation leads to MAPKs activation following downstream phosphorylation/activation of different proteins, for instance transcription factors. X residue in T—X—Y motif determines substrate specificity for MAPKs (Dong et al.,2002; Johnson and Lapadat, 2002). Different MAPKKKs and MAPKKs having unique specificity for substrates and activation kinetics can activate a certain MAPKs that allows for activation of a single MAPK through a variety of different stimuli, antigens and co-stimulatory receptors including certain cytokine receptors, FcRs and TLRs (Harnett et al.,2005; Ropert, 2005; Sweenay and Firestein, 2006).

MAPK activation is regulated through negative feedback mechanisms. For instance, not less than three classes of protein phosphatases, for instance, tyrosine phosphatases, threonine phosphatases and dual-specificity phosphatases, are induced by MAPKs that inhibit MAPKs through dephosphorylation (Jeffrey et al., 2007; Owens and Keyse, 2007). Specifically HePTP that is a tyrosine phosphatase, PP2A that is a threonine phosphatase and MKP3 that is a dual specificity phosphatase are the three MAPK phosphatases for ERK2 (Zhou et al., 2002). The dual-specificity phosphatases are a family of protein that can dephosphorylate the T–X–Y motif at both threonine and tyrosine residues leading to its inhibition (Camps et al., 2000). The dual-specificity phosphatases are regulated at transcriptional level and targeted inhibition of MAPKs is due to expression of protein phosphatases having affinity for specific substrate (Saxena and Mustelin, 2000). Procaspase activating compound (PAC-1) regulate ERK and p38 through dephosphorylation while MKP-1 is found to inhibit all types of MAPKs through negative feedback mechanisms(Thalhamer et al., 2008).

Dysregulated activation of MAPKs is linked to pathogenesis of Rheumatoid Arthritis in several studies(Paunovic and Harnett, 2013). Synovial inflammation, progressive cartilage destruction, bone erosion and angiogenesis are characteristic features of Rheumatoid arthritis and MAPKs are found guilty of involvement in each of these stages of disease (Figure 1B). Synovial tissue analysis of Rheumatoid Arthritis (RA) and osteoarthritis patients revealed MAPKs presence. Evidence of phosphorylated i.e. activated MAPKs in tissues from RA patients further established its role in the disease pathogenesis (Schett et al., 2000).

Table 1. Roles of various Signaling pathways in RA and potential therapeutic targets

Signaling Pathway	*Normal Role*	*Role In RA*	*Therapeutic Targets*	*References*
MAPK Signaling	• BCR/TCR signaling; • Survival and growth; • Apoptosis; • Proliferation; • Differentiation; • Inflammation.	• Synovitis; • Bone erosion; • Cartilage damage	• P38 MAPKs; • JNKs; • ERKs	Thalhammer*et al*., 2008 ; Li *et al*., 2013; Paunovic and Harnett, 2013
OG! Signaling	Mediating innate and adaptive immunity; Limiting inflammation; Cell proliferation	nduces expression of proinflammatory genes roduction of matrix metaloproteinases from synovial fibroblasts ecruits immune cells to the inflamed pannus	roteasomal inhibition to prevent NFkB activation nhibition of IKKs	Lawrence, 2009; Simmonds and Foxwell 2008
TLR Signaling	• Inflammation by activating NF-kB, and chemokines (TNF-, , IL-1 , IL-6) against PAMPs and DAMPs	• Synovial inflammation mediated by activating TNF-, , IL-8, IL-6, IL-15; • Destruction of bone by causing differentiation of monocytes into osteoclasts; • Increased risk of cardiovascular complications by activating MIF; • Maintenance of inflammation by expression of angiogenic factors VEGF, IL-8.	• Inhibition of adaptors (MyD88 and MAL/TRIP) to reduce cytokine and MMPs Expression; • Targeting IL-15; • Targeting MIF (macrophage migration inhibiting factor).	Ospelt*et al*., 2008; Sacre*et al*., 2007; Jung *et al*., 2007; Kim *et al*., 2007; Popa*et al*., 2006; Cho *et al*., 2007
Wnt Signaling	ell polarity; ell migration; one metabolism; ynovium proliferation, and rganogenesis	LS activation; timulate proinflammatory cytokines, and annus formation	Wnt1, Wnt5a, Wnt7b, z5 receptor, RANKL	Katoh and katoh, 2007; Miller *et al*., 1999
JAK-STAT Signaling	• Transcriptional regulation in reaction to binding of extracellular signaling molecule (cytokine, growth factors, chemokines) with the transmembrane receptor	• Constitutive STAT-3 DNA binding activity possibly caused by IL-6 leads to abnormal gene expression which is consistent with inflammation and active immunity	• Controlling Jak3 activity, inhibition of activated STAT-3; • inhibiting Jak-STAT signaling by induction of suppressors of cytokine signaling (SOCS) proteins	Darnell, Kerr, & Stark, 1994; van der Pouw Kraan *et al*., 2003; O'Shea, Park, Pesu, Borie, &Changelian, 2005; Ivashkiv& Hu, 2003

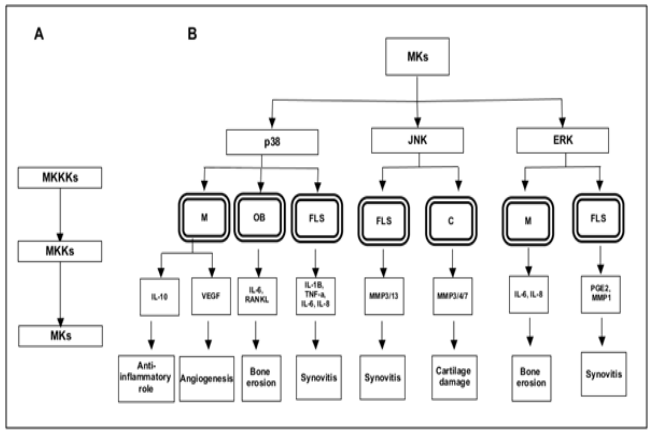

Figure 1. A. Activation cascade of MAPKs. **B.** Role of MAPKs in RA pathogenesis. MKKKs MAPK kinase kinases, MKKs MAPK kinases, MKs Mitogen activated protein kinases (MAPKs), JNK c-Jun N-terminal Kinases, ERK extracellular signal-regulated kinases, M macrophage, OB osteoblasts, FLS fibroblast like synoviocytes, C chondrocytes, IL-1B/6/8/10 interleukins, VEGF vascular endothelial growth factor, RANKL receptor activator of nuclear factor kappa B ligand, TNF-α tumour necrosis factor, MMP 1/3/4/7/13 matrix metalloproteinases, PGE2 prostaglandin E2.

Phospho-p38 MAPK is reported in synovial microvessel endothelium as well as in the synovial layer's lining. While phosphorylated and activated ERK-MAPK was found in cells residing in synovial lining and mononuclear infiltrates. Whereas activated JNK-MAPKs were found to be resident of sub lining mononuclear cell infiltrates (Schett et al., 2000). IL-6, TNF-αand IL-1 induce IL-8, Matrix Metalloproteinases and various adhesions molecules production and thus accused to be involved promoting RA in the synovium through enhancement of cell infiltration, inflammatory responses and destruction of cartilage(Thalhamer et al., 2008). IL-6, TNF-α and IL-1 activates all classes of MAPKs in synovial fibroblasts (Schett et al., 2000) while in chondrocytes, ERK, JNK and p38 MAPK were found to be activated by IL-1 and TNF-α leading to induction of Matrix Metalloproteinases including MMP-1 and MMP-13(Masuko-Hongo et al., 2004; Liacini et al., 2003; Mengshol et al., 2004). Moreover, in inflamed synovial tissue IL-1 and TNFα are reported to have a vital role in p38 and ERK-MAPK activation in vivo, as their inhibition lowered the MAPKs activation in transgenic mice expressing TNF-α (Gortz et al., 2005). Furthermore, role of these feedback mechanisms of MAPK signailing was emphasized by studies on murine, in which collagen induced arthritis abbreviated as CIA was worsen due to MAPK phosphatase-1 (MKP-1) deficiency (Salojin et al.,2006). However, reduced inflammatory responses were observed in PAC-1 (dual-specificity phosphatase) knockout mice. Further findings indicated that all MAPK classes are erroneously activated in synovial tissues and their differential activation patterns may be a sign of their involvement in the disease pathogenesis(Thalhamer et al., 2008).

All of the above mentioned reports establishes MAPKs as a potential molecular targets for therapeutic intervention for rheumatoid arthritis (Paunovic and Harnett, 2013; Li et al., 2013). In order to create safe and effective drugs, MAPKs Inhibitors are of recent research interest (Li et al., 2013). In the light of recent findings and analysis, p38 -MAPK is established as a key molecular target for an anti–proinflammatory cytokine production leading to therapy for Rheumatoid Arthritis. The α-isoform of p38 is expressed in the RA synovium and is involved in regulation of intracellular pathways of TNF-α, IL-1β, and cyclooxygenase 2 production (Malemud and Miller, 2008; Thalhamer et al., 2008). Inhibitors of p38α are reported to successfully suppress the production of TNF-α and IL-1β in monocytes and arthritis animal models (Nishikawa et al., 2003). Therefore, it can evaluated that compounds inhibiting p38 MAPK activation may have beneficial therapeutic use in autoimmune disorders including RA. Despite a speedy progress on p38 MAPK inhibitors, little evidence exists claiming it to be an effective approach in RA. An extensive research is ongoing in this area studying p38-MAPK inhibitors in RA and in other chronic inflammatory diseases are either incomplete or are in progress (Cheriyan et al., 2011; Zhang et al., 2010).

1.2. NFκB Signalling in Rheumatoid Arthritis

NFkB constitutes a family of ubiquitously expressed transcription factors which are significant regulators of inflammation and various immune responses (Abu-Amer and Faccio, 2006). NFkB is essential for cell proliferation and signal transduction for normal vertebrate development. There are five types of NFkB TFs found in mammals which include RelA (p65), RelB, c-Rel, NFkB1 (p50/p51 precursor) and NFkB2 (p52/p100 precursor). When active, different members of NFkB form different homodimers and heterodimers of any of its five subunits each activating a particular gene set. The most prevalent active heterodimer is RelA (p65) and p50 (J.A. Roman-Blas and S.A. Jimenez, 2006). A stretch of 300 amino acids known as homology domain is present in all five members of NFkB protein family that mediates dimerization, nuclear translocation of NFkB and its association with DNA (Ghosh and Karin, 2002; Carlsenet al., 2004; Bouwmeesteret al., 2004). Binding of NFkB TFs to DNA occurs at specific promoter regions known as Rel sites (Karin et al., 2004).

NFkB is primarily present in the cell cytoplasm sequestered by inhibitory kB proteins (IkB) which render it inactive (Figure 2). IkB proteins include IkBα, IKBβ, IkBγ, IkBε (Li and Verma, 2002; Ghosh and Karin, 2002).For binding to NFkB proteins, IkB proteins contain ankyrin repeats at the C-terminal. While at the N-terminal, it contains a nuclear export signal thus, controlling the shuttling of NFkB between nucleus and cytoplasm (Roman-Blas & Jimenez, 2006). Binding with IkB is crucial to retain p50-p65 heterodimer within the cytoplasm.

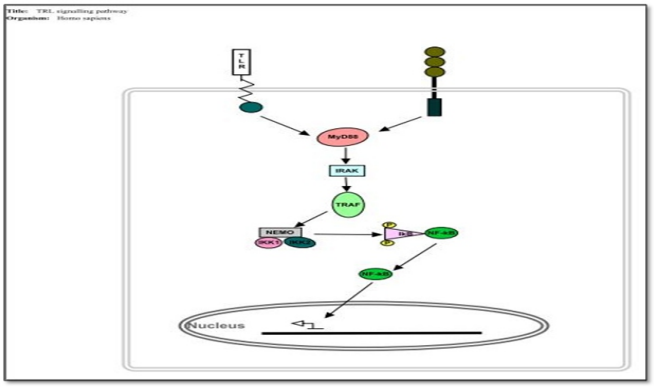

Figure 2. Upon stimulation, TLR/IL-1R initiate downstream signalling via MyD88 activating IRAK, TRAF and IKKs. IKK1 and IKK2 phosphorylate IkB leading to its proteolytic degradation and allowing translocation of NFkB into the nucleus. NFkB regulates transcription of genes involved in inflammation.

A wide range of stimuli including TNFα, IL-1, UV radiations, free radicals, bacterial and viral products which activate TNF receptors, IL-1 receptors/TLR superfamily, Nod-like receptors, B cell and T cell receptors (O'Neill, 2006) result in phosphorylation and subsequent degradation of IkB by E3 ubiquitin ligase complex and 26S proteasome. Phosphorylation of IkB inhibitors at serine/threonine residues is carried out by IkB kinases (IKK) (Roman-Blas & Jimenez, 2006) which comprise IKKα (IKK1) and IKKβ (IKK2). IkB Kinases are therefore key regulators of NFkB activity. NFkB essential modulator (NEMO), a scaffold protein, is another important regulator of NFkB activity.

NFkB signaling pathway plays multiple important functions for organismal growth and development. However, when normal functioning goes array, irregular NFkB signaling gives rise to diseases like Rheumatoid Arthritis, osteoarthritis (OA), asthma, inflammatory bowel disease, Diabetes Type 2 and cancer. p50 and p65 have been found to be abundant in RA and OA synovitis (Handel et al., 1995). Synovial tissues of RA patients have a higher number of cells producing NFkB1 at the cartilage-pannus junction than other body cells (Benito et al., 2004).

IKK2 activates the NFkB canonical pathway whereas IKK1 activates the non-canonical pathway by phosphorylating p100 and activating p52 (J.A. Roman-Blas and S.A. Jimenez, 2006). Despite of having structural similarity with IKK1, IKK2 has been found to play a dominant part in NFkB activation when fibroblast-like synoviocytes (FLS) are stimulated with cytokines (Udalova et al., 2002). Activation of IKK2 is the key to NFkB-mediated synthesis of IL-8, IL-6, and intercellular adhesion molecule 1 (ICAM-1) after cell stimulation with TNFα and IL-1 occurs (Aupperle et al., 2001). It has been shown that when a dominant negative IKK2 adenoviral construct is used to block IKK2 in vitro, synthesis of IL-1, IL-6, IL-8 and ICAM-1 doesn't occur after FLS treatment with cytokines. However, in Rheumatoid Arthritis production of TNFα is not dependent upon IKK2 (Andreakos et al., 2003).

RA is a chronic inflammatory joint disease in which synoviocytes play a significant role in causing cartilage damage by producing IL-6 and IL-8. A study showed that treatment with simvastatin inhibited IL-6 and IL-8 production from IL-1 stimulated FLS by inactivating the NFkB pathway (Lazzerini et al., 2007).

Upon reception of an appropriate stimuli, the TNF receptors recruit TNF receptor-associated death domain (TRADD), receptor-interacting protein 1, TNF receptor-associated factor 2 (TRAF2). IL-1/TLR family initiates signal transduction via MyD88, MyD88 adaptor-like (MAL), Toll/IL-1 receptor domain-containing adaptor protein inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) (O'Neill, 2006). Likewise, T cell and B cell receptors also recruit a number of signalling molecules in order to activate NFkB pathway. All these complex pathways present a challenge to develop an effective inhibitor of NFkB activation. Many selective inhibitors of IKKs and NEMO have been developed which show promise for future RA therapies.

1.3. TLR Signalling in Rheumatoid Arthritis

Toll-like receptors (TLRs) are evolutionary conserved receptors expressed on various cell types including myeloid cells, fibroblasts, epithelial cells etc. They recognize certain molecules associated with foreign beings (PAMPs) and generate response. TLR4 recognize bacterial lipopolysaccharide (LPS) using adaptor proteins MyD88, CD14 and LBP, TLR1, 2 and 6 recognize lipoproteins, TLR5 recognizes flagellin, TLR9 sense the CpG island of bacterial DNA while TLR3, 7 and 8 bind to viral DNA/RNA (Goh et al., 2012). There has also been evidence of DAMPs (damage-associated molecular patterns) i.e. molecules released by necrotic cells e.g. systolic heat-shock proteins and extracellular matrix components like fibrinogen, tenascin-C, heparin sulphate, hyaluron (HA) all of which are up-regulated in response to damage are recognized by TLRs (Goh et al., 2012).

All TLRs are transmembrane receptors which use leucine rich repeats in the extracellular domain to recognize ligands while the cytoplasmic domain has a conserved motif (Toll/interleukin (IL)-1 receptor (TIR) domain) this domain interacts with the adaptor proteins to trigger downstream signalling (Brentano et al., 2005).

Once activated by the PAMPs or DAMPs downstream signalling is brought about by various adaptor proteins; MyD88, MAL/TIRAP, TRIF, TRAM and SARM. All TLRs signal via MyD88 except TLR3 which utilizes TRIF (Santegoets et al., 2011). These adaptors activate protein kinases e.g. MAPK leading to activation of various transcription factors like AP-1, NF-kB all of which transcribe the production of inflammatory cytokines and chemokines TNF-α, IL-1β, IL-6 and interferons (Santegoets et al., 2011).

The signalling pathway can be MyD88 dependent or independent. In the MyD88 dependent pathway, the adaptor protein mediates the phosphorylation of IRAK 1 and 4 which then bind to TRAF6 activating IKK complex thereby inducing the activation of NF-kB transcription factor and MAP kinases (Brentano et al., 2005). While the hallmark of independent pathway involves the activation of transcription

factor IRF-3 consequently leading to the induction of IFN expression which involves TRIF adaptor (Brentano et al., 2005).

In rheumatoid arthritis (RA) number of studies has given evidence of the role of TLRs in the pathogenesis of the disease thereby also providing therapeutic targets for treatment. Ospelt et al (2008) determined levels of TLRs (1-10) in RA as compared to normal. TLR3 was expressed most followed by TLR4 then TLR2 while TLR10 was not detected in tissue and fibroblasts (Ospelt et al., 2008). It can be deduced that activation of TLRs occurs early leading to synovial inflammation and joint destruction. While another study by Roelofs et al (2005) showed that TLR3 and 7 were expressed in synovium in RA patients and stimulation with their respective ligands led to dendritic cell maturation and production of IL-6 and TNF-α. Expression of various TLRs was also determined by Seibl et al (2003) to investigate their regulation in RA. In both synovial fibroblasts and tissue TLR2 expression is up-regulated after stimulation with its ligand as well as cytokines IL-1β and TNF-α along with the translocation of NF-kB to the nucleus (Seibl et al., 2003).

Similarly the role of TLRs in chronic inflammation was determined using four RA patients. TLR 2 and 4 was seen to be up-regulated while the addition of their respective ligands showed marked increase in TNF-α and IL-8 mediated by adaptors MyD88 and MAL/TIRAP as determined by respectively inhibiting those (Sacre et al., 2007). This provided evidence that the adaptors were needed for cytokine production, inhibiting the adaptors also significantly reduced expression matrix metalloproteinases (MMPs) (Sacre et al., 2007) which are involved in tissue destruction. It could thus be deduced that in addition to cytokine production TLRs also regulate the destructive processes via MMPs.

Since inflammatory cytokines leading to persistent synovial inflammation are characteristic of RA, the linkage between TLR signalling and the production of such cytokines has been studied in different studies. A particular group used stimulating ligands of TLR2 and 4 to determine expression of IL-15. IL-15 is upregulated both by TLR 2 and 4 which is brought about by downstream signalling via NF-kB (Jung et al., 2007). IL-15 is linked to inflammatory diseases, when present in synovium it activates T cells, neutrophils while also reducing apoptosis, promoting proinflammatory cytokines and autoreactivity. The initiation of IL-15 by stimulation with TLR2 and 4 might be important in pathogenesis of inflammatory synovitis and targeting IL-15 might be a potential target for treatment.

Destruction of bone and cartilage in RA is brought about by the action of cytokines utilizes the interaction of various cells (lymphocytes, macrophages) to activate MMPs and osteoclast activity. Osteoclasts are a class of macrophages that degrade bone matrix, their differentiation is directed by RANKL and M-CSF. In RA, osteoclastogenesis is increased causing bone resorption and destruction. RANKL expression was shown to increase when fibroblasts were stimulated with TLR2 and 4 ligands and also partly by the proinflammatory cytokines, TNF-α and IL-1β (Kim et al., 2007) which are products of TLR signalling. TRAP-positive cells which are markers for osteoclasts were also seen after monocytes were stimulated with TLR ligands (Kim et al., 2007). Involvement of TLR signalling in osteoclastogenesis was also shown by another study which suggested that TLR3 and RANKL expression was high in RA (Kim et al., 2009). This suggests that TLR2, 3 and 4 activation enhances expression of RANKL which assists osteoclastogenesis by causing osteoclast differentiation from monocytes. The system provides a direct link between inflammation and bone erosion.

MIF (macrophage migration inhibiting factor) plays a crucial part in pathogenesis of autoimmune disorder and are incriminated to increase the risk of cardiovascular morbidity by causing atherosclerotic plaques. It was shown that dendritic cells (DC) derived from RA patients had high levels of MIF after stimulation with TLR2 and 4 (Popa et al., 2006). This increased concentrations of MIF found in synovial fluid and serum can be used as a novel marker for the disease. The same study also tested the role of cytokines on MIF secretion, TNF- α and RANKL caused an increase in MIF (Popa et al., 2006). TNF-α secreted in RA induces the production of MIF which adds on to the destructive processes by the upregulation of matrix metalloproteinases (MMPs). The cytokines play a role in bone erosion and inflammation of joints thereby suggesting that by regulation of MIF is in fact a process to augment the inflammatory loop.

Another way to maintain synovitis and leukocyte influx in RA is by angiogenesis. Angiogenic mediators include IL-8, VEGF etc. The production of both IL-8 and VEGF were markedly increased when synovial fibroblasts were stimulated by TLR2 ligand (PGN) therefore IL-8, VEGF and TLR2 were co-expressed and resulting downstream signalling occurred via MyD88 dependent pathway (Cho et al., 2007). The synovium of RA patients is infiltrated by numerous cells at the site of inflammation and angiogenesis provide the route of transmigration therefore it plays a key role in mediating inflammation at early stages.

Pathogenesis of RA is complex with many components not all of which are elucidated. One pathway might include the dysregulation of unfolded protein response (UPR) to increased number of destructive synovial fibroblasts. One component of UPR is the XBP1 which is a transcription factor regulating genes involved in ER stress. TLR2 and 4 are implicated in splicing of XBP-1 in absence of ER stress and leading to production of inflammatory cytokines IL-6 and TNF while expression of spliced XBP-1 was seen in blood cells of patients with RA (Savic et al., 2014). However the full implications of this dysregulated pathway in RA disease pathogenesis still needs to be understood fully.

Evidence from the studies on animal models and cell cultures highlight the role of TLRs in RA pathogenesis, targeting these receptors and their signalling pathway might provide an effective therapeutic tool for treatment. It is also interesting to consider the extent with which each TLR interacts to bring about the pathological changes. RA is a heterogeneous disease with unpredictable therapy response therefore, there is need of novel biomarkers to aid in personalized medicine for effective therapy.

1.4. Wnt Signalling in Rheumatoid Arthritis

Wnt signaling pathway acts as an antique and evolutionarily preserved pathway that switches basic features of neural imitating, cell polarity, cell immigration, cell fate determination and embryonic organogenesis escalation (Komiya & Habas, 2008; M. Sen, 2005). Wnts are secreted glycoprotein (Wnt1, Wnt2, Wnt2B, Wnt3, Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt8A, Wnt8B, Wnt9A, Wnt9B, Wnt10A, Wnt10B, Wnt11 and Wnt16) that bind with frizzled (Fz) receptors and may also necessitate Low-density lipoprotein receptor-related protein 5/6(LRP 5/6) receptor that is in case of canonical pathway (Habas &Dawid, 2005; Li et al., 2012).

Wnts glycoprotein and Fz receptor multifaceted activates many down signaling including non-canonical β-catenin-independent pathway (Wnt/Ca2+ pathways and Planar Cell Polarity (PCP)) or canonical and Wnt/β-catenin pathway (Yamaguchi, 2001). In Canonical pathway Wnt1, Wnt3a, Wnt8 and non-canonical pathway Wnt5a, Wnt11 most of the time enrolled as Wnt modulator (Figure 3)(Kühl, 2004). Wnt signaling is essential for tissue maintenance that why Wnt signaling involves in human genetic disorder and other diseases like absence of limbs (Niemannet al., 2004), cancer (especially Familial Adenomatous Polyposis), and autoimmune diseases like Rheumatoid arthritis, lupus erythematosus (Kim et al., 2010).

Here we focus Wnt pathway in the development of RA. Current studies have revealed Wnt signaling pathway implicate in the pathogenesis of RA. Beta-catenin is principal element in the instigation of canonical Wnt pathway. B-catenin express high altitude in RA patient and also up regulates FLS expression (Kuanget al., 2009). Similarly down signaling in non-canonical Wnt pathway ignition through activation of Ca+2 mediated enzymes calcineurin (CaCN), protein kinase C (PKC) and calmodulin/Ca2+ dependent kinase II (CamKII) (Sheldahl, Park, Malbon, & Moon, 1999).

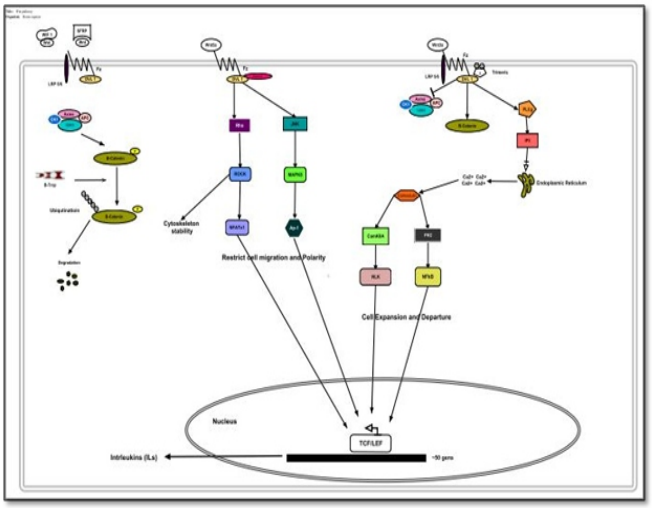

Figure 3. Wnt pathway mediated gene expression (left) canonical Wnt pathway that is mediated by LRP LDL related protein and activated by Wnt proteins. (middle) non-canonical Wnt pathway activation (left) Ca2+ dependent Wnt pathway that down signalling facilitated by IP3 inositol triphosphate, Ca2+, CamkIICa2+/Calmodulin-dependnet kinase II, NLK Ser/Thr-protein kinase, CaCN Calcineurin, result in activation of nuclear factors TCF/LEF T-cell specific transcript/lymphoid-enhancer binding factor.

Previous study confirmed that wnt5a express by FLS in RA patient. Wnt5a triggers PKC signaling cascade which further stimulate NFkB pathway, as a result stimulation of IL genes. IL genes are IL-15, IL-6 which are diagnostic marker in patient with chronic RA (Wilson, Szabo, & Salzman, 1999). So, IL-6 and IL-15 fabrication as result of Wnt-5/Fz5-mediated signaling by the RA FLS may cause pannus formation, cartilage destruction, and bone erosion. It has been reported that ani-Fz and anti-Wnt5a were blocking their activity(Aveleira, Lin, Abcouwer, Ambrósio, & Antonetti, 2010; Malini Sen, Chamorro, Reifert, Corr, & Carson, 2001). So, Wnt5a/Fz5 has potential as a therapeutic target. Wnt5a silencing through SiRNA technology also reduces the expression in RA patient (Katoh & Katoh, 2007).

RANKL expression was testified at vigorous bone erosions sites. Normal expression of RANKL in osteoblasts and stromal cells under the stress of hormones (calcitriol, PTH, prolactin, estrogens, glucocorticoids), growth factors (BMP, oncostatin M, TGFb, IGF1, PDGF) and cytokines (PGE , IL-1, IL-6, IL-17, TNFa) (Lorenzo, Horowitz, & Choi, 2008). RANKL production in RA is activated by synovial fibroblasts, macrophages, Th17 cells, activated B cells and dendritic cells (Okamoto & Takayanagi, 2011).Another approach to treat RA is overcome the expression of RANKL by using anti-Fz5. We can also obstruct osteoclastogenesis and bone erosion in RA pathogenesis (MaliniSenet al., 2001).

Wnt1-mediated signaling pathway show dynamic role in the development of RA. Wnt1-mediated signaling controlled the fibronectin manifestation in RA FLS through canonical Wnt signaling pathway. Wnt1-mediated signaling must endorse the articular cartilage impairment (Nakamura, Nawata, & Wakitani, 2005).

Wnt1 include significantly involved in Wnt7b elevated expression in the cartilage of osteoarthritis and synovium of RA patients. Wnt7b increased the pro-inflammatory cytokines expression IL-1B, TNF-α and IL-6 under FLS transfection. Wnt signaling pathway signifying that Wnt7b upstream factor also act as an imperative role in the pathogenesis of RA (Fouque-Aubertet al., 2012).

MiRNA validation assays and sequencing approve impairment of miR-146am miR-155 and miR-223 are related with RA patient, similarly other MiRNA that are miR-323-3p and miR-221/222. Bioinformatics analysis revealed that elevated level of miR-323-3p also associated to activation of Wnt pathway. miR-323-3p is used as diagnostic marker (Pandiset al., 2012).

1.5. The JAK-STAT Pathway in Rheumatoid Arthritis

Owing to the side effects caused by various immunosuppressive drugs being used in rheumatoid arthritis, research has been prompted in a different orientation (Walker & Smith, 2005). A molecule that is restricted to particular immune cells can serve as a target in designing immunosuppressive drugs with reduced toxicity. Protein

tyrosine kinase; Janus kinase (JAK) appeared as potential target. Cytokines have a central role in immune cell regulation (O'Shea, Pesu, Borie, & Changelian, 2004).

Cytokines belonging to the hematopoietin family bind to Type I and Type II cytokine receptors and initiate a signal cascade inside the cell by the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway. A number of such cytokines have been reported/are theorized to have a role in RA (e.g., interferons, IL-6, IL-2, IL-7, IL-12, IL-15) and therefore, a detailed understanding of JAK-STAT activation within the rheumatoid synovium will have a direct impact on the discovery and development of novel therapeutic agents for the disease (Walker & Smith, 2005).

JAKs are activated; auto and trans-phosphorylates by the cytokine binding with the receptor. These receptors are then phosphorylated by JAK to generate sites for docking of molecules that effect signaling. Signal transducers and activators of transcription (STATs) are the family of molecules that are significant for conducting cytokine signals and regulating gene expression (Darnell Jr, Kerr, & Stark, 1994).

Most of the JAKs bind several cytokine receptors and are widely expressed. JAK3 is specifically expressed, has narrow tissue distribution and interact particularly with one cytokine receptor subunit, making it a potential therapeutic target (Kawamura et al., 1994). Six cytokines bind to a common cytokine receptor subunit named γ-subunit (γc). The cytokines that selectively binds and hence activate JAK3 are: Interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 (O'Shea, et al., 2004). Phosphorylated sites on cytokine receptors are identified by STAT and other molecules which are then recruited and activated by JAK driven tyrosine phosphorylation. STAT on activation disassociates from the receptor, form dimers in the cytoplasm which are then translocated to the nucleus where they bind to the γ -activated site (GAS) enhancers (Figure 4)(Walker & Smith, 2005).

Mononuclear cells isolated from RA synovial fluid (SF) showed integral STAT-3 DNA binding activity, and revealed that STAT-3 in control human monocytes was efficiently activated by the soluble factors present in SFs of RA patients (Sengupta, Chen, Zhong, Darnell, & Ivashkiv, 1995)(Wang, Sengupta, Zhong, & Ivashkiv, 1995). Immunohistochemistry was used to detect activation of tyrosine phosphorylation of STAT-3 in synovial tissue of RA patient in vivo. It is not certainly known that which cytokine is responsible for the activation of STAT-3 during synovial inflammation in RA in vivo, but IL-6 is the possible candidate (Ivashkiv & Hu, 2003).

STAT-3 activation and increased expression of STAT-1, STAT-4, and STAT-6 had been described in RA synovitis. (Fruchtet al., 2000)(Müller-Ladner et al., 2000). However, it was not sure whether these STATs were able to control gene expression and phenotype of synovial cell, in short can perform their function fully or not, in synovitis. STAT function can be assessed by the measurement of the STAT-dependent genes expression. Van der PouwKraan and coworkers used the same approach and used microarray technique to produce an ample profile of gene expression in RA synovium (van der Pouw Kraan et al., 2003). This significant study outspreads information obtained from former gene expression profiling experiments in RA, and illustrates two patterns showed by RA tissues. The first group showed gene expression regular with active immunity and inflammation, with significant expression of genes of antigen-presenting cell and lymphocytes, along with genes encoding transcription factors, activation markers, signaling molecules, chemokines, and cytokine receptors. The second group of RA tissues had gene expression profile more alike to osteoarthritis tissues. These tissues expressed genes imperative in tissue remodeling and showed less expression of immune and inflammatory genes (Ivashkiv & Hu, 2003)(van der Pouw Kraan, et al., 2003).

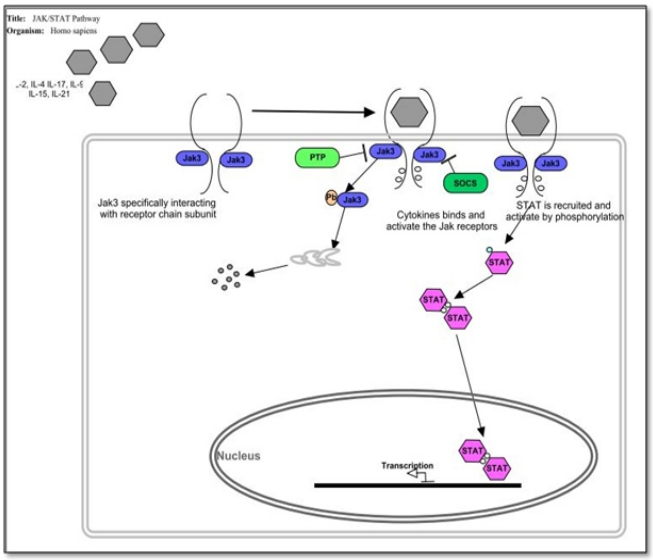

Figure 4. JAK-STAT pathway in Rheumatoid arthritis

These anomalies in gene expression in the diseased state may either be variant in various classes of RA, or specific to different stages in disease course, characterized by varied activity and ultimately leading to rigorously damaged joints with reduced inflammation in a final "burned out" stage (Ivashkiv & Hu, 2003)(van der Pouw Kraan, et al., 2003).

Closely related DNA sequences are recognized by the different STATs (Ivashkiv, 1995), and it has been shown (Nakajima et al., 1996) that STAT-3 can trigger expression of some of the "STAT-1–dependent genes". STAT-3 is essentially activated in RA synovium which further supports the concept of a likely role for STAT-3 in activation of STAT-dependent genes in these tissues. Also there is indication that "STAT-1 dependent genes" can be triggered to be expressed in a STAT-3–dependent way by IL-6, along with an unidentified SF factor. (Sengupta, et al., 1995).

As JAK3 is selectively expressed in specific tissues, it was suggested that interrupting with Jak3 function could be the basis for a novel class of immunosuppressants. Furthermore, because the Jak3 results in immunodeficiency but not pleiotropic defects, an exceedingly specific Jak3 inhibitor should also have precise restricted and specific effects. JAK3 targeting contrasts with broadly used immunosuppressive drug, which shows non-particular targeting and have varied

side effects. In standard, Jak3 inhibitor selection would have benefits over the recent agents (O'Shea et al., 2005).

Another strategy includes initiation of suppressors of cytokine signaling (SOCS) proteins which fade experimental arthritis. This illustrates the role of STATs in pathogenesis of rheumatoid arthritis (Ivashkiv and Hu, 2003). Inhibition of JAK-STAT signaling by SOCS family members can be done by inhibiting catalytic activity of JAK, consequently inhibiting receptor docking sites for STAT and targeting cytokine receptors for deprivation by proteasomes (Walker and Smith, 2005). Therefore SOCS can assist as drug against rheumatoid arthritis via inhibiting STAT activity and ultimately RA pathogenesis

2. CONCLUSION

Various important signalling pathways when become aberrant, as described in this review, could manifest disease pathogenesis as it occurs in Rheumatoid Arthritis (Table 1). Disarrayed signalling pathways present a possible target therapy for RA. Blocking signal transduction by using gene therapy, peptide inhibitors and small interfering RNA techniques to target important signalling components is being exploited for the treatment RA. However, as these signalling molecules are involved in various other overlapping pathways important for metabolic function, a more plausible option is prevention of receptor activation to block a specific pathway. For the development of such inhibitors there are still a number of challenges to overcome in order to develop effective therapeutic drugs for RA.

Conflict of Interest

All authors have no conflict of interest regarding this manuscript for publication.

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Helicobacter pylori Virulence Regulatory Network: Insights into the Host-Environment and Pathogen Interactions

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ABSTRACT

Helicobacter pylori (*H. pylori*) has evolved significant regulatory mechanisms in order to acclimatize in extreme gastric environment of human beings. The virulence machinery of H. pylori is complicated as virulence factors of pathogen not only interact with transcription and translational machinery of host, but also are involved in the progression and development of the disease. The present study is an effort to model virulence mechanism in H. pylori, particularly ferric uptake regulator (FUR) under acidic and iron (Fe) depleted conditions, as well as its effects on the well known virulence factors cytotoxin-associated gene A (cagA) and vacuolating cytotoxin A (vacA) gene. The virulence regulatory network of cagA and vacA is modeled based on an asynchronous kinetic logic formalism introduced by René Thomas. The cagA-vacA virulence regulatory network is then elaborated qualitatively to obtain insights into H. pylori induced pathogenesis. The findings have revealed the significant regulatory pathways through which H. pylori spreads infection to the gastric cells, and also verified that cagA is associated with acute gastritis while vacA is involved in vacuolation, apoptosis and atrophy. Interestingly, both cagA and vacA were found to modulate each other virulence potential which ultimately leads to the state of chronic gastritis; which in turn drives the pathway smoothly towards gastric adenocarcinoma via the formation of pre-malignant lesions. The proposed strategy can be extended to understand the mechanism of other similar bacterial infections and disease progression. It will also help in the prioritization of potential therapeutic targets to control such serious infections.

Key words: Helicobacter pylori, CagA, Virulence, Adenocarcinoma, Regulatory networks.

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) affects more than 50% of the world's population and the infection is more prevalent in developing countries (~90%) in comparison to the developed world (~20%). However, in most of the cases, the infection remains asymptomatic (Blanchard, et al., 2004). *H. pylori* infection is the primary cause of chronic gastritis which is recognized as one of the risk factor for the development of gastric carcinoma. Besides other gastric pathologies like gastric and peptic ulcerations and lymphomas, *H. pylori* associated gastritis also leads to gastric cancer (about 70% globally) (Peek and Blaser, 2002). Gastric cancer is amongst the most common cancers and second leading cause of cancer related deaths, worldwide (Ferlay, et al., 2010). Therefore, the pathogen is classified as Class I carcinogen by WHO (Humans, 1994). Inside the host, *H. pylori* can live in extreme conditions such as gastric acidity and iron limitation. In order to cope with such conditions, the pathogen has evolved specific but rather limited set of transcriptional regulators (Salama, et al., 2000, Baltrus, et al., 2009). These transcriptional regulators are superior to their homolog proteins found in other bacteria in terms of their additional roles besides their traditional functions. For example, ferric uptake regulator (Fur) is one of the most important global transcription regulator (Delany, et al., 2001, Delany, et al., 2001, Alamuri, et al., 2006, Gilbreath, et al., 2012, Pich, et al., 2012) which facilitates bacterial pathogen to acclimatize in acidic and iron limiting conditions (Bereswill, et al., 2000). In most of the bacterial species, including *H. pylori*, the Fur exists in the form of a complex (Fe-Fur), which binds to the promoters of iron uptake genes and regulates their transcription. However, Fur in *H. pylori* has acquired the ability to bind the promoter of other host genes independent of the complex (Apo-Fur) and regulates the transcription process (Bereswill, et al., 2000, Delany, et al., 2003, Ernst, et al., 2005). This dual property of Fur broadens its regulatory control on the expression of genes, which in turn equip the bacterium to adapt in environment with low iron levels.

This study focuses on the dual property of Fur to regulate the genes which can help *H. pylori* to survive and colonize in acidic environment and nutrient limited conditions for several years, ultimately leading to inflammatory responses and resulting in significant clinical consequences like ulceration and gastric neoplasm (Blaser & Parsonnet, 1994). It is, therefore, necessary to understand the biology of *H. pylori* infection, not only because it is causative agent of adenocarcinoma but also can be a representative model of effects of chronic inflammation on gastric mucosa. (Blaser & Parsonnet, 1994).

Virulence machinery of *H. pylori* is quite complicated and

various interacting factors (virulence factors and host factors) are involved in the progression and development of disease (Atherton, 1998, Höcker & Hohenberger, 2003, Kusters, et al., 2006). This developmental behavior and the dynamics of the pathogenesis vary in the presence of different environmental cues (such as low pH, iron availability etc.). No doubt, the interplay of various key players in the pathogenesis of *H. pylori* complicates the prediction of exact virulence mechanism. Recent advances and development of high-throughput techniques in computational and system biology enhanced our understanding of such complex biological systems. These techniques have greatly contributed in the accumulation and growth of quantitative and qualitative biological information, which leads to the prediction of biological interfaces at different levels (Saadatpour & Albert, 2013). In order to target single outcome or behavior, it is necessary to identify the virulence factors and re-construct the whole or representative virulence regulatory network (VRN) (Karlebach & Shamir, 2008).

The kinetic logic based method builds biological regulatory network (BRN) exploiting threshold response. The biological entities (genes or proteins) interact with each other either positively or negatively. In other words, certain concentration of a particular entity can either increase or decrease the activation of other entity. Thus two types of biological regulation i.e. either positive regulation or activation and negative regulation or inactivation are the essential interactions between genes or/and products of BRN. Both types of biological regulation are a function of sigmoidal curve (Ahmad, et al., 2012).Thus our study studies the pathogenic nature of H. pylori in gastric carcinogenesis pertaining to the regulatory expression of cagA and vacA, this regulatory expression along with other associated factors is referred to as virulence regulatory network (VRN), which has been constructed by logical modeling based on Kinetic logic formalism and with the help of existing experimental evidences. The results generated may show various regulatory pathways in the form of a state graph. These pathways form a network in which pathological outcomes (states) will be produced because we expect differential expression of virulence factors under different environmental stresses. These states may cross each other and regulate other states to produce an outcome; in case of *H. pylori* that is gastric carcinogenesis.

2. METHODOLOGY

2.1. Network modeling

In network modeling, the components are shown as nodes, while interactions are represented by edges. These interactions could be of two types; positive and negative symbolizing activation and inhibition of the nodes, respectively. In biological systems, large number of components including genes, proteins and their corresponding levels leads to a complex interaction networks. Translating the structure and dynamics of such networks is the primary step in understanding the overall behavior of the cell (Karlebach & Shamir, 2008). To target a single outcome and behavior, such network modeling techniques can be modified to include specific nodes and interactions and study the respective structural and dynamical analysis to uncover the underlying specific biological organization.

The network modeling strategies can be broadly classified in two types; dynamic modeling and discrete modeling. Dynamic modeling takes nodes as molecular species and considers the effect of time on the population levels of species. It observes and describes how the rate of population levels changes with respect to time. These models are illustrated by set of differential equations and are more reliable in extracting the dynamical behavior of biological system. Whereas discrete modeling e.g. Boolean models, finite state logical models and Petri nets are helpful in providing qualitative description with few or no parameters (Saadatpour & Albert, 2013).

René Thomas in 1970 proposed Boolean logic method for the discrete modeling of biological regulatory network (BRN) with the corresponding qualitative modeling of dynamical behavior of system. However, René Thomas rendered this method limited because this method uses only two levels 0 and 1, which is not sufficient to encompass other types of problems. Subsequently René Thomas modified the Boolean logic to Kinetic logic and demonstrated its practical reliability by applying to different gene regulatory networks, which is adopted successfully in our previous studies (Ahmad, et al., 2012, Paracha, et al., 2014).

2.2. Prediction of *H. pylori* Virulence Regulatory Network (VRN)

The general overview of pathogenesis of *H. pylori* was performed through extensive literature survey and consulting through virulence factor databases such as (VfDB (Chen, et al., 2005) and VirDB (Mazzoleni, et al., 2003), which includes the identification of related virulence factors (genes, proteins and mRNAs), nature of interactions between them as well as the global regulations by transcriptional factors (Figure 1).

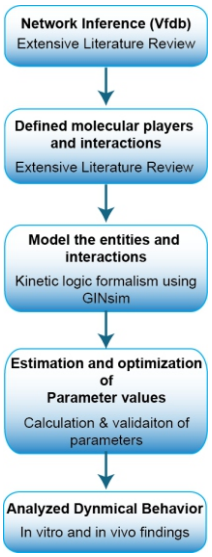

Figure 1. Outline of the Proposed Methodology. The workflow represents the schematic representation of the methodology used in the modeling of cagA-vacA VRN. The stages in this study overlap at variouspoints, which helps in refining (model reduction) the outcome of predicted VRN.

2.3. Discrete Modeling of Proposed CagA-VacA VRN

The cagA-vacA VRN was modeled using Gene Interaction Network simulation (GINsim), a computational tool that is used for the modeling and simulation of gene regulatory networks. GINsim is a java-based platform, which can be employed in qualitative modeling and analysis of biological regulatory networks. It includes graphical user interface (GUI) which helps user to draw and edit the predicted regulatory network (Gonzalez, et al., 2006). It also allows to stabilize the regulatory graph with the inclusion of logical parameters (observations) and a simulation engine to help establish qualitative dynamic behavior of the corresponding VRN (Gonzalez, et al., 2006). The tool is freely available (http://www.ginsim.org/) and is based on well-known logical formalism first introduced by René Thomas (Gonzalez, et al., 2006).

This logic known as kinetic logic is based on following definitions (Gonzalez, et al., 2006):

- **Logical Regulatory Graph:** This type of graph illustrates the regulatory interaction between genes and their products.
- **State Transition Graph:** This type of graph constructs the relative dynamical behavior from the given regulatory graph/network for the presumed initial (starting) states.

2.4. Construction of cagA-vacA VRN as Logical Regulatory Graph

With the graphical user interface (GUI) of GINsim, the regulatory graph can be created, edited and saved in "ginml" extension. Within the workspace of GINsim platform, genes can be added and interactions (activation and inhibition) can be drawn by selecting the option 'node' and 'arc' from drop down menu under the tab 'edit'. Following nodes were added into the cagA-vacA VRN; two virulence factors, i) cagA ii) vacA, one global regulator iii) fur and two environmental factors iv) low pH (acid) and v) iron limitation (Fe) as shown in Figure 2. The interactions between genes were created by adding an arc (edge) and drawing it from the node (activator/inhibitor) to another node (to be 'activated'/'inhibited' node). Modeling attributes can be added by clicking on a desired node; Id, name, checking or unchecking the input status and maximum expression level may be added. Here, all nodes were attributed maximum expression value "1" while all edges were assigned threshold value "1".

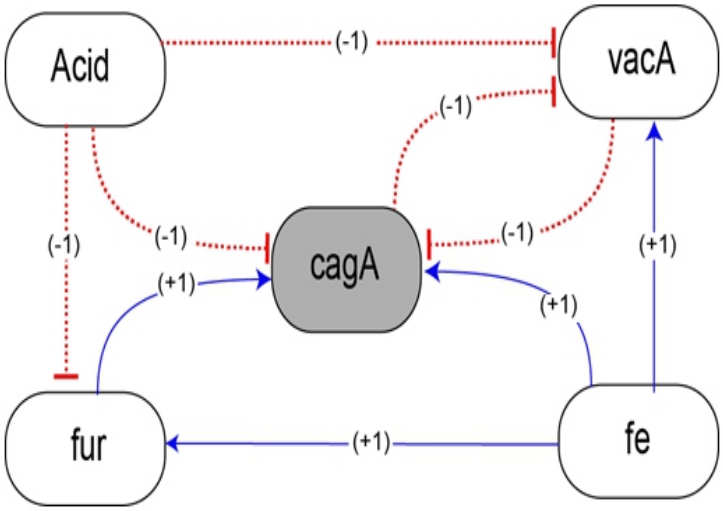

Figure 2. Logical regulatory graph of cagA-vacA Virulence Regulatory Network. The VRN shows the regulation of two important *H. pylori* virulence factors, cagA and vacA in a high acidic gastric niche and iron limiting conditions. Under simultaneous conditions of low pH and iron limitated cagA, vacA and fur are repressed. As the concentration of Fe increases, it binds to the Fur that inducts the positive regulation of cagA and vacA. CagA and VacA modulate each other activity by selectively inhibiting one another. The signs "+" (blue) and "-" (red) represent activation and inhibition, respectively. The integer "1" represents the qualitative threshold of an interaction.

2.5. Computation and Optimization of Parameter Values

For the prediction of dynamical behavior of cagA-vacA VRN, it is necessary to define parameter values or logical values for each node in VRN. For each node, the corresponding logical parameter allowed the qualitative specification of the effects of any combinations of incoming interactions (Gonzalez, et al., 2006). The total number of K parameters for each node/entity is calculated by a formula 2n where 'n' represents the number of interactions (either negative or positive) coming towards node. In GINsim, the K parameters of desired node can be added by clicking on the individual and combined interactions coming towards a particular node. The general format of a K parameter is Kentity,{resources}, where resources are the set of activators (positive regulator) when they are present (at level 1) and inhibitors (negative regulator) when they are absent (at level 0) at any instant of time. The K parameters are defined by giving either a value '0' or "1" (representing expression levels). The K parameter Kentity, {resources}=1, increase the expression of the entity to 1 when its present level is 0. Similarly, The K parameter Kentity,{resources}=0, decreases the expression of the entity to 0 when its present expression level is 1. If the entity level is equal to its parameter value then entity may not evolve. The complete sets of parameters of all entities along with their experimental evidences are given in **Table 1.**

Table 1. Logical parameters of cagA-vacA VRN. The logical parameters (K parameters) adjusted for each entity and employed in GINsim to modelled the cagA-vacA VRN.

Entity	No. of Interactions (n)	Total No. of possible K parameters (2^n)	K parameters associated with entity	Reference studies
CagA	4	2^4=16	$K_{cagA}\{\ \}=0$, $K_{cagA}=0$, $K_{cagA}\{Acid,Fe\ \}=1$, $K_{cagA}\{Acid,Fur\}=1$, $K_{cagA}\{Acid,VacA\}=0$, $K_{cagA}\{Acid,Fe,Fur\}=1$, $K_{cagA}\{Acid,Fe,VacA\}=1$, $K_{cagA}\{Acid,Fur,VacA\}=0$, $K_{cagA}\{Acid,Fe,Fur,VacA\}=1$, K_{cagA}(Ferlay, *et al.*)=1, $K_{cagA}\{Fe,Fur\ \}=1$, $K_{cagA}\{Fe,VacA\ \}=0$, $K_{cagA}\{Fe,Fur,VacA\}=1$, $K_{cagA}\{Fur\ \}=1$, $K_{cagA}\{Fur,VacA\ \}=0$, $K_{cagA}\{VacA\ \}=1$	(Merrell, *et al.*, 2003, Gupta, *et al.*, 2011, Raghwan & Chowdhury, 2014)
VacA	3	2^3=8	$K_{vacA}\{\ \}=0$, $K_{vacA}[Acid]=0$, $K_{vacA}\{Acid,CagA\}=0$, $K_{vacA}\{Acid,Fe\}=1$, $K_{vacA}\{CagA\}=0$, $K_{vacA}\{CagA,Fe\}=0$, K_{vacA}(Ferlay, *et al.*)=1, $K_{vacA}\{Acid,CagA,Fe\}=1$	(Merrell, *et al.*, 2003, Bury-Mone, *et al.*, 2004, Gupta, *et al.*, 2011, Raghwan & Chowdhury, 2014)
Fur	2	$2^{2=}4$	$K_{Fur}\{\ \}=0$, K_{Fur} (Ferlay, *et al.*)=0, $K_{Fur}\{Acid\ \}=0$ $K_{Fur}\{Acid,Fe\}=0$	(Raghwan & Chowdhury, 2014)

2.6. Construction of State Graph

After cagA-vacA VRN was constructed the state graph was generated by running a command 'run simulation' under the 'actions' menu. The interface of state transition graph includes detailing of initial states, choosing between synchronous and asynchronous construction strategy. It allows user to choose either width or depth first search algorithm for the exploration of state graph extracted from the stabilized and parameterized regulatory graph. The construction strategy chosen for cagA-vacA VRN was asynchronous and depth first search algorithm was selected. Next limits on depth and number of states can be optimized; however, this option was not used in our cagA-vacA VRN. After the generation of state transition graph, stable state(s) were identified. In generated state graph, a stable state or sink, depicts the entire system assembly, terminal point and where it cannot progress to the subsequent state, while starting state confers to a state from which all initial trajectories are originated (Ahmad, et al., 2012). States other than stable state(s) leading to and terminating at sink(s) are called trajectories. It is property of a system to achieve stability at any moment, the system is disturbed from its stable state or sink it falls back or moves to another stable state.

2.7. Analysis of the State Graph

The state transition graph using GINsim simulation tool usually comes out as complex mesh of network, which is usually hard to analyze. This state transition network can be sorted out manually by first separating out stable state(s) and then logically arranging the remaining trajectories. The Graphviz tool is graph visualization software, which represents structural data as diagrams of predicated graphs and networks in a systemic and hierarchical manner (Ellson, et al., 2002).This tool is available freely on http://www.graphviz.org/.

3. RESULTS

3.1. Prediction of H. pylori Virulence Interactome

In order to have general overview of genes involved in H. pylori pathogenesis and the prediction of H. pylori VRN, extensive literature survey was conducted and different protein regulators were predicted that are involved in global regulation of the pathogenic genes (factors) (Figure 4). These included Fur, ArsS-ArsR system, CsrA and NikR. Among the 17 transcriptional regulators of H. pylori (Danielli, et al., 2010) fur which codes for metallo-regulatory protein ferric uptake regulator 'Fur', was the first identified transcriptional regulator necessary for the growth and colonization of pathogen in gastric mucosa under acidic and iron limiting conditions. Additionally it also has influential control the expression of metabolic and energy production related genes (van Vliet, et al., 2003, van Vliet, et al., 2004). Protein Fur further expands the spectrum of its regulation by playing its dual role as iron complex form of Fur (Fe-Fur) as well as apo form of the enzyme (apo-Fur) (Ernst, et al., 2005). Fur (Fe-Fur and apo-Fur) regulates the related expression by binding to Fur box sequences located inside the promoters of metabolic genes. (Pich, et al., 2012). In, apo-form Fur regulates the expression of outer-membrane proteins (ompB and oipA), iron storage protein (pfr) and most important virulence factors (toxigenic genes); cagA and vacA while in Fe co-factored-form, Fur regulated the expression of flagellar genes (flbB and flaE) and genes implicated in iron homeostasis like frpB1,exbB2, fec1and fec2 (Ernst, et al., 2005). Free Fe is also capable of regulating the expression of several genes like vacA, cagA and other cag pathogenicity island genes (cag3, cag4 and cag26), outer-membrane proteins (hopA and omp6), fecA3 (involved in iron uptake) and transposition regulatory protein (tnpB) (Ernst, et al., 2005). ArsS-ArsR system like Fur also regulates the expression of genes involved in acid resistance (amiE and amiF; amidase and formamidase enzymes), urease operon, exbB2, dnaK operon (dnaK, hrcA and grpE; involved in heat shock responses), napA, omp6 as well as fur (Pflock, et al., 2006), sabA which in involved in adhesion to gastric cells(Goodwin, et al., 2008). ArsS-ArsR system can regulates its own expression as well (Pflock, et al., 2006). NikR is a metal responsive regulatory protein involve in nickel homeostasis (Contreras, et al., 2003). In the presence of excess nickel it upregulates the expression of nixA, a high-affinity nickel-transport protein (nixA represses the expression of urease operon) (Contreras, et al., 2003). While NikR represses the transcription of iron uptake and storage genes (pfr, fur, frpB4 and exbB/exbD), genes involved in motility (flaA and flab), genes implied in stress responses (hrcA-grpE-dnaK) and genes encoding outer-membrane proteins (omp6, omp11, omp31, omp32 and hopZ) (Contreras, et al., 2003). CsrA- carbon storage regulator, is a post-transcriptional regulator which functions as global regulator of genes involved in the acid induction of napA (gene encoding neutrophil activating protein), cagA, vacA, the urease operon (Important in colonization of gastric cells and acid resistance) and fur (Barnard, et al., 2004). It also regulates the heat shock responses of napA, groESL and hspR and the expression of ahpc (involved in anti-oxidant activity) (Barnard, et al., 2004). In addition to this, another transcriptional regulators HspR auto regulates its expression as well as regulates the expression of dnaK (Thompson, et al., 2003). Another transcriptional repressor HrcA auto regulates its expression in heat shock responses (Thompson, et al., 2003).

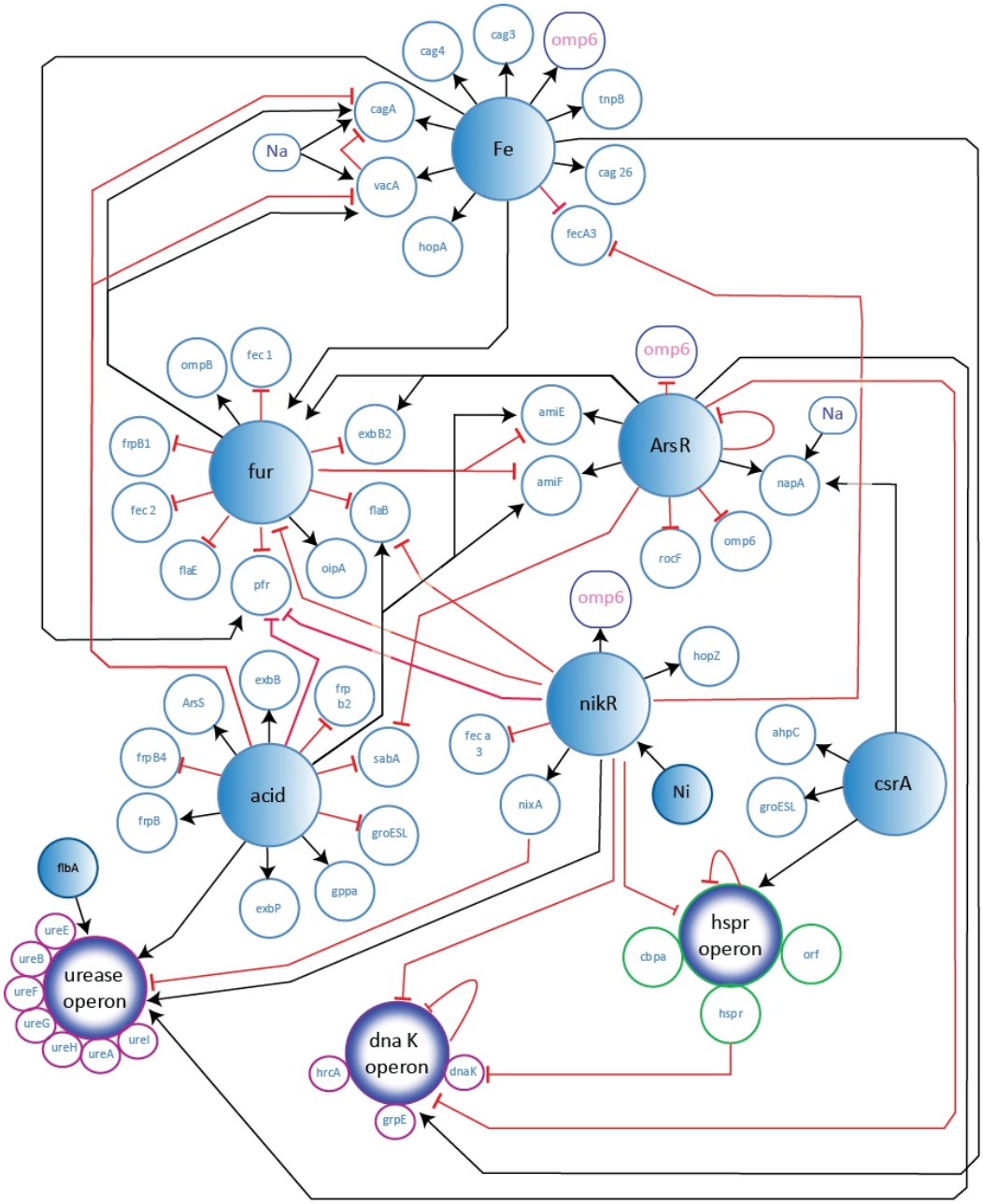

Figure 3. Derived extensive interactive network of virulence factors of H. pylori. The figure displays the detailed and complex interactions of various virulence factors of H. pylori .The network shows the differential expression of acid responsive genes, iron uptake genes, flagellar genes in acidic, iron depleted and salt conditions and their regulation by global regulators like Fur, csrA and two component systems like ArsR-ArsS system. All of the interactions were confirmed using reported laboratory findings however, the regulation of mentioned virulence factors except cagA and vacA were not confirmed through qualitative modeling. The objective of this study was to reveal the strategy that H. pylori employs to survive and persist in human gastric mucosa. There are number of components (gene and gene products) that are involved in the regulation of pathogen's virulence however, it is difficult and non-logical to include all of these components in a logical regulatory model. Therefore, such networks are constructed only to grasp the general overview of pathogenesis of bacteria. With this network in the background, only the most crucial virulence factors (cagA and vacA) of H. pylori were identified and included in the virulence regulatory graph, which was the modeled quantitatively. Such construction of a network structure was crucial for the prediction of virulence regulatory system of H. pylori. Due to complex and huge size of this network, intuition alone was not adequate to grab the dynamical behavior of H. pylori induced pathogenesis. Therefore, the next logical step was the prediction of specific virulence factors and their respective interactions. The interaction dynamic values assist in the generation of dynamical behavior of the targeted outcome of H. pylori induced pathogenesis. The straight arrow represents the activation while arrows with red dots show the inhibition of the subsequent genes.

The H. pylori colonizes highly acidic environment of stomach to adapt these harsh habitat the pathogen down regulates nine outer-membrane protein (OMP) related genes; hopD, hopA, homA, hopO, sabA, hofH, hopQ, horL, hopK and up regulates two genes horA and hp1467 (Mahdavi, et al., 2002). Acid activates ureA, ureB, ureF, ureG, ureH and ureI of urease operon. All these genes encode for urease enzyme as well as other genes involved in buffering of acidic environment are up-regulated. These genes include amiE and amiF encoding amidase and formamidase respectively (Merrell, et al., 2003). motB and hp1192, responsible for motility are also up-regulated in response to low pH (Bury-Mone, et al., 2004) e.g. genes encoding flagella rotation protein (Bury-Mone, et al., 2004) and flab which is involved in motility (Merrell, et al., 2003). On the other hand, acid (in stomach) also induce regulation of of genes involve in iron homeostasis (pfr, exbB, exbP, frpB, frpB2, frpB4), heat shock response (groESL, gppa and grpE), adhesion (sabA) and transcriptional regulators (fur and arsS component of ArsS-ArsR system (Merrell, et al., 2003)).

Interestingly, CagA and VacA are found in functional association. Protein CagA down regulates the vacuolating activity of VacA, similarly VacA reduces the activity of CagA. Studies have shown that CagA reduces the vacuolating and apoptotic effects of VacA, whereas, VacA reduces the formation of 'humming bird phenotype' induced by CagA. However, no genetic linkage is yet confirmed between vacA and cagA. Such association is reported only in host cell signal transduction. The antagonizing effect of both these entities prevents the gross damage to gastric epithelial tissues, helping the pathogen to modulate the secretion of its virulence factors and thus enable it to adapt to host cell environment (Argent, et al., 2008). The net VacA and CagA antagonizing effect on gastric epithelial tissue vary widely between strain types. Further insight into the genetic as well as molecular aspects associated with VacA and CagA signal transduction in host cell can provide better understanding of physiological interaction between the two important cytotoxins of H. pylori (Palframan, et al., 2012).

The GINsim tool is used for the qualitative (discrete) modeling of VRNs according to René Thomas formalism; it considers VRN as a directed graph along with logical parameters and in result generates the state graph, where stable states, cycles and acyclic paths between any states can be identified.

3.2. Logical Parameters (K) of cagA-vacA VRN

The logical parameters for the VRN were computed by the formula as discussed in methodology. These logical parameters were computed for the entities used to construct original cagA-vacA regulatory graph; these entities were cagA, vacA and Fur respectively (Figure 2). Table 1 represents logical parameters of each entity present in cagA-vacA VRN.

3.3. State Graph Generated from cagA-vacA VRN

The state graph was obtained after the optimization of logical parameters of biological regulatory network. The state graph encompasses all the feasible qualitative states or configurations. Each state portrays distinct expression of an entity at a particular moment of time. Moreover, each single state shown in a state graph is a combination of entities assigned in the original VRN. The cagA-vacA VRN shows each state as the qualitative expressions of cagA, vacA, Fur, Fe, acid respectively. The state graph of our VRN encloses 14 states including one stable state (Figure 3). These states were verified and explained in connection to the support from previous laboratory findings.

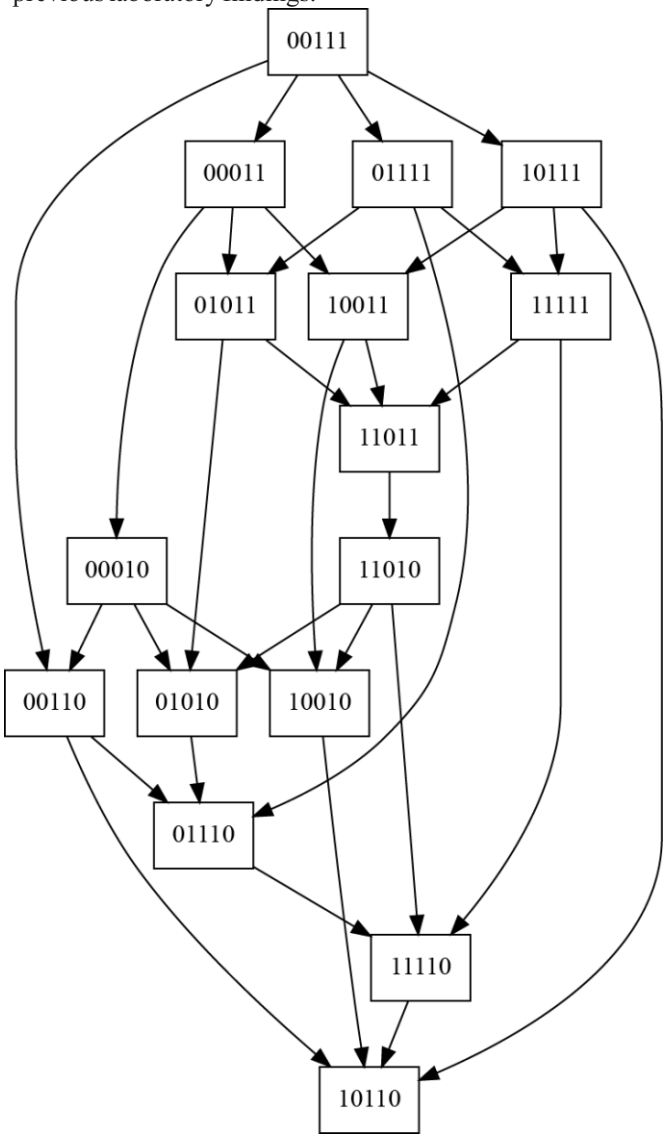

Figure 4. State transition graph of cagA-vacA VRN. State graph or of Virulence Regulatory Network helps in prediction of regulatory pathways that are further confirmed by reported laboratory findings. The cagA-vacA VRN depicts the regulatory pathways implicated in the achievement of stable state, gastric carcinogenesis through the combination of virulence factors cagA, vacA and regulatory protein Fur in acidic and iron depleted environment of gastric mucosa.

3.3.1. Analysis of the State Graph (States and their Transitions)

The starting state of the cagA-vacA VRN (state graph) is 00111. This state indicates the activation of Fur under iron limiting and low pH conditions it also indicates the condition when H. pylori adheres to the gastric epithelium of gastric mucosa (Raghwan & Chowdhury, 2014). After the adherence (00111), there are three separate states (00011, 01111 and 10111) emerging simultaneously and ultimately leading to the deadlock state (10110). The path involves the state transition from 00011 to 10110 i.e. mucosal damaged condition to the development of adenocarcinoma. For instance, the expression of cagA and vacA is immediately inducted separately in two of these states (01111 and 10111) after the adherence process. The state transition from 00111¬¬ to 01111 depicts the ongoing gastric colonization after adhering properly to gastric epithelium. Meanwhile vacA and fur are expressed simultaneously as both are extremely important factors for early successful colonization of gastric niche (Merrell, et al., 2003, Gancz, et al., 2006, Oldani, et al., 2009, Miles, et al., 2010, Pich, et al., 2012).

In an adhered state, gene vacA become activated in a Fe-Fur dependent manner while cagA merely by Fur (apo-Fur). It has been observed that absence or inactivation of Fur leads to impaired colonization conditions (Merrell, et al., 2003). Simultaneously the state transition 00111 to 10111 is crucial for pro-inflammatory responses after successful adherence. Gene cagA is induced in the host colonized epithelium which then promotes pro-inflammatory and anti-apoptotic effects since vacA (promotes apoptosis of the gastric epithelial cells) is shutdown in this state (Oldani, et al., 2009). The continued pro-inflammatory actions of state (10111) lead to the inflammation of gastric lining (11111) i.e gastritis. This inflammation is however not detrimental since vacA is switched-on at this state (11111) dilutes the disastrous effects of cagA induced inflammation and damage (Ruggiero, et al., 2006). This state will logically lead to more stable and long-lasting state (11011) clinically known as chronic gastritis (chronic inflammation of gastric mucosa also referred as chronic gastritis).

The chronic gastritis is pre-disposition to the atrophic gastritis, long-lasting ulcers that eventually results in cancer (Roesler, et al., 2011). Meanwhile, the state 01111 assists in building the state 01011, which ultimately leads to the state 11111. The state 01111 signifies the enhanced activation of vacA in the absence of its inhibitor cagA which rolls up in exaggerated vacuolating action of toxin and hence vacuolation of gastric epithelial cells. The increasing vacuolating toxicity (01111) results in extensive apoptosis of gastric cells which further advances to the immunomodulatory condition (Roesler, et al., 2011), atrophic gastritis (Israel & Peek, 2001) (11011) through early maintenance of the disease (10011) (Roesler, et al., 2011) and chronic gastritis (11011). The long-lasting and chronic conditions of gastritis (11011 and 11010) in conjunction with muted T-cell response acts as key precursors in the formation of premalignant lesions (Leung, et al., 2004) known as intestinal metaplasia (10010). The intestinal metaplasia (10010) through dysplasia finally falls into the stable state of gastric carcinogenesis (10110). No recent study suggests the clear correlation of gastric adenocarcinoma with other types of cancers therefore gastric adenocarcinoma has been assigned a deadlock or stable state of agA-vacA VRN as shown in Figure 5 (de Martel, et al., 2008, Koshiol, et al., 2012).

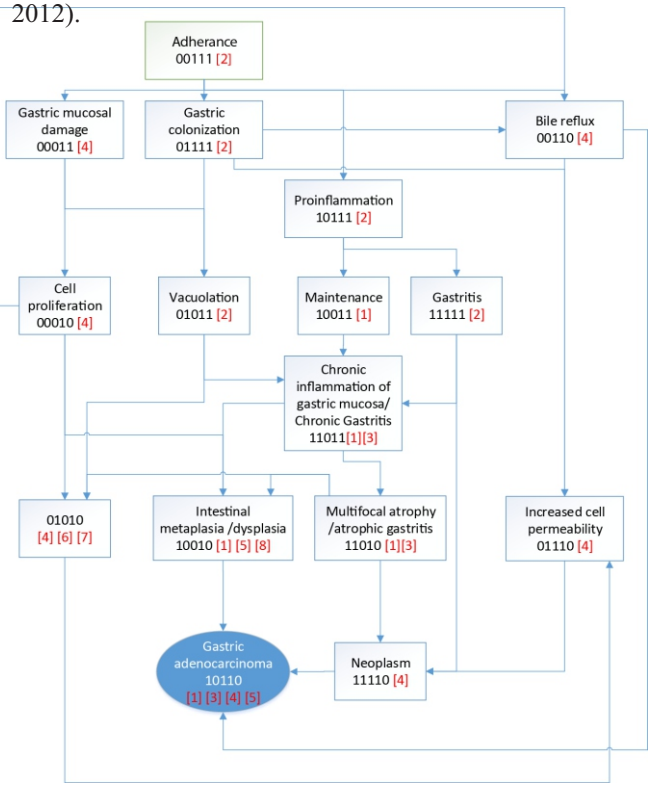

Figure 5. Schematic representation of the cagA-vacA state transition graph. The stable state of the graph is represented by state 10110, which is a potential EVENT of gastric carcinogenesis (Sobala, et al., 1993, Cahill, et al., 1994, Lynch, et al., 1995, Ricci, et al., 1996, Warburton, et al., 1998, Oldani, et al., 2009, Roesler, et al., 2011).

Adherence (00111) of H. pylori to gastric cells can cause gastric mucosal damage (00011). This results in the repression of cagA and vacA under low pH conditions which lead to expression of other gene which are not discussed here as they are not included in cagA-vacA VRN and are involved in epithelial damage. After gastric mucosal damage (00011), altered cell proliferation (Lynch, et al., 1995) is observed in gastric cells (00010). Gene cagA does not have any effect on cell proliferation while vacA specifically inhibits cell proliferation (01010). It has been proved in previous studies that increase in altered cell proliferation is not associated with the severity of gastritis rather it is the direct effect of the pathogen (Ricci, et al., 1996). Cell proliferation is now considered as one of the earliest changes in mucosal lining and acts as catalyst in the development of adenocarcinoma (Cahill, et al., 1994). Several studies have proved this development as abrupt increase in mucosal cell proliferation which leads to the accumulation of neoplastic clone of cells (11110) especially in the presence of bile reflux (00110) and chronic epithelial damage (00011) (Lynch, et al., 1995). Bile reflux (00110) also known as 'reflux' or chemical ' gastritis'

increases the cell permeability (01110). The presence of these agents (00110, 00011 and 01010) contributes synergistically to the development of intestinal metaplasia and is consistent with the laboratory findings. It is a well-known fact that intestinal metaplasia and dysplasia always proceed before the development of neoplasm, however, intestinal metaplasia/dysplasia may or may not proceed towards neoplasm. The neoplastic clone of cells along with other etiological agents (11111, 11010 and 01110) establishes the stable state of gastric adenocarcinoma /gastric carcinogenesis (Lynch, et al., 1995).

The model is usually confined to target a single behavior or outcome. The shortest possible route toward the normal sink was also identified and used as the reference trajectory (path) as shown in Figure 6. The small divergences from this trajectory that culminate at the stable state have been discussed.

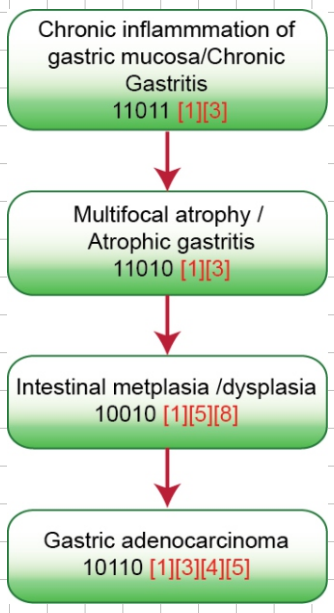

Figure 6. Shortest route to stable state. The shortest possible route to adenocarcinoma is derived, which is in agreement with the clinical findings, epidemiological data and prospective histo-pathological studies.

3.3.2. Analysis of the State Graph with Respect to pH and Iron Conditions

The inflammation is usually inducted for the provision of nutrients to H. pylori species thus Fe=1 in the following states; pro-inflammation (10111) leads to acute gastritis (11111) followed by chronic gastritis (11011) until it reaches atrophic gastritis (11010) because continued inflammation is not very favorable for the survival of the pathogen. Thus reduction of inflammatory conditions leads to the stable, immune-compromised and premalignant states of atrophic gastritis (11010) and intestinal metaplasia/dysplasia (10010) (Blaser, 1995).

Increased acid production is evident in inflammatory states (10111, 11111, and 11011) (Blaser, 1995, Israel & Peek, 2001, Leung, et al., 2004), while decreased acid production or hypochlorhydria (acid=0) are the characteristic of atrophic cells (atrophic gastritis/11010) and premalignant lesions (intestinal metaplasia/10010).

4. DISCUSSION

A cell, whether prokaryotic or eukaryotic, responds to external stimuli by the coordination of various biological processes. These biological pathways include interactions as well as regulation of gene, protein, signal transduction or metabolic process. For instance, at gene level, various transcriptional regulatory proteins regulate either self-expression or activity of other genes. The repression or activation of respective genes by these regulatory proteins leads to either increase or decrease in number of mRNA transcripts. This ultimately changes the concentration of respective proteins, which leads to new state or new behavior of the cell. All these genes and their products constitute a dense network of interactions in which one component interacts either positively or negatively with other components. In a miniature world of such myriad components and their intricate interactions in biological systems, network modeling assists in representing the entire system in cohesive outline (Karlebach & Shamir, 2008).

H. pylori - a persistent pathogen is able to resist and flourish in acidic environment of the stomach and under iron limiting conditions. Our proposed cagA-vacA VRN suggested the pathway, that leads to the gastric carcinogenesis, emerges by the combination of fluctuating expressions of cagA, vacA and Fur under low pH and iron depleted conditions. Nearly all individuals colonized by H. pylori have co-existing gastric inflammation; however, only a small percentage of colonized individuals follow the proposed sequela. Potential risks are due to the combinations of variable expression of bacterial gene products to differences in the intensity and magnitude of host inflammatory response to pathogen or distinct interactions between host and the pathogen (Israel & Peek, 2001).

The pathogen after adhering successfully to the host cells can provoke a series of inflammatory conditions causing gastric mucosal damage and gastric colonization. Unlike other bacteria (e.g. Mycobacterium tuberculosis) which inhibit its host for many years but remain mainly in dormant or latent form, H. pylori cause persistent and continuous inflammation (Cadamuro, et al., 2014). Such chronic and long-lasting conditions of inflammation can lead to the development of atrophic gastritis and formation of pre-malignant lesions such as intestinal metaplasia, dysplasia and neoplasm, which altogether create the fate of gastric carcinogenesis.

The Fur has been found to play critical role in productive gastric colonization; however, it is certainly not required for persistent infection (Gancz, et al., 2006). Therefore, the events following adherence and gastric colonization, does not require fur expression. VacA is another important factor in successful colonization of gastric mucosa. Colonization of the pathogen initiates strong systemic immune response which promotes the chronic inflamed environment of gastric mucosa, chronic gastric inflammation or chronic gastritis through enhanced expression of cagA for its preparation in pro-inflammatory effects (Israel & Peek, 2001). The early maintenance of disease and co-expression of cagA and vacA

in persistent gastritis drives gastric carcinogenesis. The vacA, in absence of interference from cagA, promotes vacuolation and subsequent apoptosis of the gastric cells. The cagA expression along with vacA counteracts the damaging effects of vacA, which is harmful for host and also for the survival of H. pylori The co-expression of both toxigenic genes also assists establishing the state of chronic inflammation, which is a risk factor for the gastric malignancy. According to few studies, the acid production increases in a state of chronic inflammation, most likely results from the increase in serum gastrin acid and decreased somatostatin levels caused by gastric inflammation/gastritis (Israel & Peek, 2001).

In a state of chronic inflammation, there is a remarkable increase in cell apoptosis. The ability of H. pylori to alter the apoptosis influences its associated clinical outcomes of disease. The enhanced apoptosis accelerate the process of cell demise and hence contribute to the atrophic gastritis with increase in the risk of gastric carcinoma. In contradiction, the reduced rates of apoptosis especially in the presence of hyper proliferation catalyze the retention of mutagenized cells which in turn make H. pylori infected individuals liable to the gastric cancer. The atrophic gastritis is often accompanied by intestinal metaplasia (Ohkuma, et al., 2000, Israel & Peek, 2001).

The H. pylori induced injury by inflammatory cells not only inducts DNA damage but also promotes the production of radicals that are responsible for accumulation of mutations and malignancies. The pathogen thus provides a breeding ground for the formation of pre-neoplastic and neoplastic lesions through acute and chronic gastritis (Guarner, et al., 1993). Cell proliferation induced as a direct effect of pathogen is regarded as the pioneer inducer of mucosal changes in the development of gastric adenocarcinoma. In the normal mucosal environment, the undifferentiated cells undergo active cell proliferation in the gastric pits and neck region of the glands. These newly formed cells after maturation and differentiation migrate to the surface of mucosa and arrange themselves in columnar cell layers at lumen. Studies have confirmed that the highest rate of cell proliferation is at the base of gastric pits in healthy mucosa. However in H. pylori infected mucosa, particularly in case of H. pylori associated atrophic gastritis, there was enhanced cell proliferation throughout the entire gastric pits (Cahill, et al., 1994). The pathogen also inhibits the secretion of ascorbic acid entering the stomach thus lowers the gastric juices ascorbic acid levels. It has also been proved that eradication therapy can restore the normal ascorbic levels in gastric juices. Bile reflux has been proved to play supporting role in the H. pylori induced gastric adenocarcinoma due to its carcinogenic properties; however, there is no recorded data available that proves the origin of bile reflux due to H. pylori infection. Several studies have been conducted to confirm the link between gastric colonization and bile reflux in patients that were undergone through gastric surgery. The results have shown that postoperative 'chemical gastritis' play an important role in the eradication of H. pylori. Furthermore, it was proved that H. pylori again colonize (Ladas, et al., 1996) the gastric mucosa after bile diversion surgery. Despite of all the above observations, H. pylori and bile reflux co-exist as the bile acid samples collected from patients that are not undergone through gastric recession are much lower in concentration than collected from post-operative gastric samples; this proves that H. pylori species are able to withstand the harmful effects of bile reflux in intact stomach.

Bile reflux also promotes the enhanced cell permeability, which in turn expose the nuclei of gastric epithelial to the mutagens present in lumen. This leads to the mutagenesis preparing for the final stage of H. pylori related disease outcomes i.e. gastric carcinogenesis (Lynch, et al., 1995). The carcinogenic nature of bile reflux along with chronic epithelial damage and ever-increasing cell proliferation leads to the accumulation of mutations, under pathological and physiological stress the mature gastric tissues start to decrease in number and immature cells begin to increase. Two independent studies have shown that the increasing risk of gastric cancer depend upon these two pathways; 1) vacA expression in gastric epithelial cells causing vacuolation and subsequent apoptosis and 2) the increasing cell proliferation in intestinal metaplasia and dysplasia supported by cagA expression. Increasing cell proliferation that is not balanced by the synergic increase in apoptotic indices over years of colonization support the carcinogenic ability of cagA+ strains towards increasing risk for gastric carcinogenesis (Israel & Peek, 2001). The ongoing combination of stimuli leads to the buildup of neoplastic cell clones that eventually become malignant and drive the pathway to gastric carcinogenesis.

5. CONCLUSION

The pathogenic nature of H. pylori and its adaption to the stressful environment of gastric mucosa is attributed to the regulation and differential expression of virulence factors by few specific transcriptional regulators. Our proposed study highlighted the differential expression of Fur and its influence on the expression of cagA and vacA in addition to low pH and iron levels. All of the above-mentioned conditions assist in constructing various regulatory pathways that help to attain the ultimate disease outcome, gastric carcinogenesis. The high-grade inflammation is inducted by cagA under hyperactive secretion of acid while vacuolation, apoptosis and atrophy mediated by vacA under hypo secretion of acid. The expression of both genes helps to regulate and modulate the damaging consequences in the formation of long lasting disease conditions like chronic gastritis and atrophic gastritis. These conditions assist the formation of pre-malignant lesions through increased cell permeability and in combination with aberrant cellular proliferation that ultimately contributes in the developmental of carcinogenesis. The proposed cagA-vacA VRN has presented in simplified form to understand the pathogenesis and regulation of two virulence factors by global regulator Fur under low pH and limited availability of iron. However, many other virulence factors may influence the regulatory pathway of gastric pathogenesis like ureA, ureB, ureF- ureI, napA, ferric iron transport genes including frpB1, frpB2, feoB etc. Moreover, the genes implicated in acid acclimation and iron deficiency is regulated by other regulators in addition to Fur. The proposed cagA-vacA VRN may not applied be to all H. pylori strains and hence cannot be considered as thumb rule for the ultimate pathogenic nature of H. pylori.

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EDITORIAL

Withdrawal of a paper

The corresponding author Gul Majid Khan, E-mail: drgulmajeed@yahoo.com requested the following refered research paper authored by Asif Nawaz, Shafaatullah Shah, Kifayatullah Shah, Hashaam Akhtar, Asim-ur-Rehman, Abid Hussain, Gul Majid Khan. Curcumin: Its Pharmacological and Therapeutic Properties published in NUST Journal of Natural Sciences (NJNS), 02 (02): 20-23 (2012) to be considered withdrawn. According to corresponding author the published work was a part of the M-Phil research thesis of his student who is also the first author of the paper. It has been communicated to the editor that the student had submitted the said paper without prior permission and the according to the corresponding author the said paper also had borrowed material from another paper they have already published in another journal in 2011 [Curcumin: A natural Product of Biological Importance, Gomal University Journal of Research, 27(1): 07-14 (2011] , and even the reference of that article has not been cited in the current paper. Keeping in view the above facts and figures, the request of the corresponding author of the paper and supervisor of the first author, the said paper is thereby cancelled and considered widrawn from NJNS as per norms of the journal.

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