ABSTRACT
Myelodysplastic syndromes (MDS) are clonal disorders characterized by ineffective hematopoiesis and peripheral cytopenias [1] and reported as hematological malignancy of all age groups including childhood. Generally, morphological examination of marrow smear remains the cornerstone of diagnosis of MDS. In ambiguous cases, a cytogenetic evaluation is suggested to correlate morphological findings and confirm diagnosis. While, the diagnosis of MDS is also complemented by cytogenetics [2], there are no cytogenetic abnormalities specific or consistent for any morphologic subgroup of MDS. Chromosomal abnormalities would ultimately lead to the discovery of the genetic lesions important in the pathogenesis of MDS. Existing therapeutic options for MDS patients include supportive care, low/high-intensity therapy. However, therapeutic dilemmas exist because of disease heterogeneity and patient age. Hence, development of an effective therapeutic protocol inevitably requires additional investigations in understanding the initial genetic event of the presumed multi-step pathogenesis of MDS.

Molecular studies suggest that pathogenetic lesions in MDS relate to ineffective hematopoiesis and enhanced apoptosis of hemopoietic cells within MDS marrow. Hence, signaling molecules and the pathways involved in mediating apoptosis was extensively analyzed and a hypothesis was developed that in addition to pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-beta (TNF-β) and interferon-gamma (INF-γ), transcription factors like NF-κB also involve/influence the pathogenesis of MDS [3]. It has been demonstrated that polymorphisms in genes of cytokines might have direct functional significance by altering level of genes expression and/or its function in many types of cancer [4]. Studies have shown that majority of polymorphisms found in cytokine genes and their receptors are located in the promoter, intronic and 3' untranslated regions and polymorphic studies in the same genes often shown conflicting results and affecting the prognostic outcome in many patients [5]. Hence, for a better understanding on pathogenesis of MDS and therapy objectives are derived as follows:
➢ To evaluate the level of apoptosis and chromosomal aberrations in bone marrow of MDS-RA patients.
➢ To analyze expression of pro-inflammatory cytokines, nuclear transcription factor levels and
➢ Study polymorphisms in the genes of cytokines and nuclear transcription factors which regulate the apoptosis pathway.

This thesis comprises of six chapters. The first chapter summarizes about MDS, its pathology, classifications, and molecular pathogenesis in general and for MDS-RA in detail to formulate those objectives. The second chapter describes in detail on the materials and methods used to study apoptosis, chromosome morphology, expression profiles and polymorphisms in cytokines in the study populations, namely MDS-RA (n=35) at presentation, leukemia (n=10) and healthy individuals (n=40). The study was carried out with prior medical ethics committee clearance from Sri Ramachandra University (Ref: MEC/06/51/22) and Government General Hospitals, Chennai (GO no: 33937/E1/2008-1 and MEC-45487/ME1/1/2008) from where the samples were collected. The apoptosis was analyzed in the bone marrow and peripheral blood samples using Annexin V apoptosis detection kit (Abcam). Mononuclear cells were isolated and treated with Annexin V antibodies, as per the manufacturer’s protocol. A total of 200-500 cells (as per availability) were scored for each subject and the apoptotic cells were calculated in percentage.

Cytogenetic analysis was performed on all the bone marrow (BM) samples by short term culture without any stimulant and peripheral blood cultures with mitogen. The samples were cultured in RPMI 1640 media with fetal bovine serum (FBS) and incubated at 37°C in 5% CO₂ incubator. Harvesting, slide making and banding were carried out according to standardized protocol [6]. About 20 metaphases from each sample were karyotyped according interpreted as per ISCN [7]. Fluorescence in situ hybridization (FISH) with locus specific probes (7q31 and 5q31) was employed for samples failed/inconclusive cytogenetics. About 200-500 interphase nuclei were scored
under fluorescent microscope using the appropriate filters and interpretation was done according to ISCN [7].

The expression profile of cytokines, *TNFα*, *TNFRI*, *TNFRII*, *TGF-β1*, *TGF-β2*, *TGF-β3*, *Fas-L*, *Fas-R*, *INF-γ* and transcription factors *NF-κB1*, *NF-κB2*, *NF-κB3* were studied using Real-time PCR (RT-PCR) with SYBR green protocol (ABI). The RNA was isolated using Trizol reagents and cDNA synthesis using high cDNA reverse transcription kit (Applied Biosystems). *GAPDH* expression was used as endogenous control for all the reactions. ΔCt values for all the cytokines were estimated by calculating the difference in Ct values of endogenous control and the respective cytokines.

To study the polymorphism, DNA was isolated using QIAamp DNA blood mini kit (Qiagen). Single nucleotide polymorphisms (SNP) of *TNF-α*-238, -308(G/A), *TGFβ1* -1347(C/T), 28(T/C), 73(G/C), 11089(C/T), 20743(C/T), *TGFβ2* 23267(G/T), 36158(C/T), 41537(C/G), 59941(A/G), 69153(C/T), *TGFβ3* -614(C/T), 4875(G/A), 15101(G/A), 17369(T/C), 17682(A/G) and *INF-γ* 261(A/T) were studied [8]. The PCR products were analyzed in 2% agarose gels to calculate allele and genotype frequencies.

The mean value of apoptosis, expression profile of cytokines and genotype frequencies were compared for statistical significance using SPSS software version 17.

The third chapter presents the results of apoptosis obtained among the three population groups. The mean percentage of apoptotic cells obtained in control, MDS-RA, and leukemic group were 23, 47, and 10% respectively. Thus, the results reveal a significantly higher (*p<0.001*) apoptotic cells in MDS-RA group than control; this suggest that the microenvironment of the bone marrow is unfavourable for blast cells contributing to the marrow and peripheral cytopenia. However, lower rate of apoptosis in leukemic group when compared to that of control and MDS groups, attributes to pathology of the disease.
Cytogenetic analysis in MDS group of disorders has become a routine due to its significant role in prognostication. Hence, the fourth chapter describes the cytogenetic profile of each group and compares the findings between three study groups. The obtained results show a normal karyotype in control samples; however, in MDS-RA group only 2 out of 35 patients (6%) shows abnormalities and 3 out of 10 (30%) samples revealed abnormal karyotype in leukemic group. The abnormal karyotypes in MDS-RA group are isochromosome 17q and deletion of 7q. There were no culture failures in both control and leukemic group but 3 cases in MDS-RA group had failed culture; it was attributed to the very low cell count in the sample due to severe cytopenia. Failed cytogenic cases were subjected to interphase FISH to evaluate abnormalities of chromosome 5/5q31 and 7/7q31 and all showed a normal pattern.

The fifth chapter discusses the cytokines and transcription factors expression profiles obtained from RT-PCR results. Expression of cytokine was considered higher if the Δct value is lower and vice-versa. Expression profile of all cytokines shows significant difference between the control and MDS-RA group as TNF-α (p<0.01), TNFRI (p<0.001), TNFRII (p<0.001), TGF-β1 (p<0.001), TGF-β2 (p<0.01), TGF-β3 (p<0.01), Fas-R (p<0.001), INF-γ (p<0.003). Similarly, expression of transcription factors NF-κB1, NF-κB2, NF-κB3 shows a significant increase in MDS-RA than that of control groups. However, there was no significant difference in the expression level Fas-L between MDS and control group. When a similar comparison were made between MDS-RA and leukemic groups, expression levels of all cytokines and transcription factors are significantly low in the latter. The significance of the different levels of cytokines and the nuclear transcription factors were discussed in detail in the understanding of MDS-RS pathogenesis.

The sixth chapter reports the allele and genotype frequencies of TNF-α -238, -308(G/A), TGFβ1 -1347(C/T), 28(T/C), 73(G/C), 11089(C/T), 20743(C/T), TGFβ2 23267(G/T), 36158(C/T), 41537(C/G), 59941(A/G), 69153(C/T), TGFβ3 -614(C/T), 4875(G/A), 15101(G/A), 17369(T/C), 17682(A/G) and
INF-γ 261(A/T). Of the various SNP’s studied, AT heterozygote of INF-γ 261(A/T) and GG homozygote of TGFβ1 73(G/C), shows a significant association towards MDS-RA pathogenesis. INF-γ, AT polymorphism was observed in 48% of controls and 85% of MDS-RA group. This indicates that this genotype significantly high in MDS-RA (OR-5.53; CI – 1.58 to 19.30; p<0.01). Similarly, G/G genotype of TGF-β1 was observed in 54% of the MDS-RA and 13% of controls (OR - 10.13; CI – 1.94 to 52.90; p<0.01).

References