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CICLO XXV

Role of microRNAs in the regulation of normal T cell development and pathogenesis

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SOMMARIO

Diversi casi di leucemia linfoblastica acuta delle cellule T (T-ALL), una delle neoplasie associate a cellule T, nascono da timociti immaturi corrispondenti alle fasi iniziali di maturazione. Diversi sottotipi di T-ALL mostrano traslocazioni cromosomiche che coinvolgono gli elementi del promotore dei geni che codificano i recettori delle cellule T e fattori di trascrizione potenzialmente importanti nel processo di oncogenesi. Più recentemente, la deregolazione di microRNA, geni e fattori di trascrizione associati al normale processo di maturazione delle cellule T è stata indicata come ulteriore meccanismo coinvolto nella patogenesi delle cellule T. Per comprendere il ruolo dei microRNA nella regolazione dello sviluppo normale delle cellule T e nella patogenesi, sono state effettuate un'analisi bioinformatica su un dataset di timociti normali e una meta-analisi su una dataset di T-ALL. Nei timociti normali, è stata osservata una tendenza generale di up-regolazione dei microRNA nei timociti Singolo-Positivi (SP) rispetto ai Doppio-Positivi (DP), ad indicare che l'up-regolazione di questi microRNA potrebbe causare la down-regolazione dei geni associati a un fenotipo immaturo. Diversi microRNA differenzialmente regolati tra SP e DP sono risultati essere coinvolti nel differenziamento cellulare, nella sopravvivenza cellulare e nell'apoptosi. La meta-analisi sul dataset di T-ALL ha identificato diversi fattori di trascrizione up-regolati in T-ALL rispetto ai timociti normali. Tra questi, abbiamo trovato alcuni fattori di trascrizione up-regolati indipendentemente dal sottotipo di T-ALL che potrebbero interagire con fattori di trascrizione specifici di sotto-tipi per causare leucemogenesi delle cellule T. Tre microRNA sono down-regolati sia nel fenotipo immaturo DP e in T-ALL: hsa-miR-22, hsa-miR-26a e hsa-miR-132. Questi microRNA potrebbero avere un ruolo nella regolazione di geni e fattori di trascrizione connessi con fenotipo immaturo di cellule T normali e nella leucemogenesi. È interessante notare che hsa-miR-22 è stato predetto avere come target ETS2, un fattore di

trascrizione up-regolato e coinvolto nella maturazione dei timociti. In sintesi, l'analisi bioinformatica suggerisce che microRNA, geni e fattori di trascrizione coinvolti nello sviluppo e nella fisiologia delle cellule T normali possano essere deregolati nel processo di trasformazione neoplastica e contribuire all'insorgenza di T-ALL.

ABSTRACT

Several cases of T cell Acute lymphoblastic leukemia (T-ALL), one of the neoplasms associated with T cells, arises from immature thymocytes corresponding to initial stages of maturation. Different T-ALL subtypes have chromosomal translocations between promoter elements of the genes of T cell receptors and oncogenic transcription factors. More recently, the deregulation of microRNAs, genes and transcription factors associated with normal T cell development has been suggested as a further mechanism involved in T cell pathogenesis. In order to understand the role of microRNAs in the regulation of normal T cell development and pathogenesis, bioinformatics analysis was performed on a normal thymocytes dataset and a meta analysis was performed on a T-ALL dataset.

In normal thymocytes, a general trend of up regulation of microRNAs was observed in Single Positive (SP) compared to Double Positive (DP), indicating that miR up-regulation could be important for down-regulating genes associated with an immature phenotype. Several differentially regulated microRNAs between SP and DP were found to be involved in cell differentiation, cell survival and apoptosis.

Meta analysis on T-ALL dataset identified several up regulated transcription factors between T-ALL and normal thymocytes. Among these, we found few transcription factors up regulated irrespectively of T-ALL subtypes that may interact with sub type specific transcription factors to cause T cell leukemogenesis. Three microRNAs are down regulated both in immature phenotype DP and in T-ALL: hsa-miR-22, hsa-miR-26a and hsa-miR-132. These microRNAs may have possible roles in up regulation of genes and transcription factors associated with immature phenotype of normal T cell and in leukemogenesis. Interestingly, hsa-miR-22 was predicted to target ETS2, an up regulated transcription factor involved in thymocyte maturation. In summary, the bioinformatic analysis suggests that microRNAs, genes and

transcription factors involved in the regulation of normal T cell development and physiology may be deregulated in the process of neoplastic transformation and contribute to the onset of T-ALL.

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1. INTRODUCTION

Investigating the cancer genome is one of the most challenging tasks that oncologists are facing today. Different high throughput techniques like gene expression profiling, microRNA expression profiling, SNP genotyping, DNA methylation profiling, Copy Number variation profiling and deep sequencing technologies are being used to decode the hidden aspects of cancer development and progression. Since microRNAs have emerged as future diagnostic and prognostic tools in many different tumors, oncologists around the world frequently use microRNA and gene expression profiling to classify and study cancer. Role of microRNAs in regulating normal development of hematopoietic cells, epithelial cells, ectoderm cells are also reported in scientific literature. MicroRNAs are also known to regulate different transcription factors (TFs) and genes during neoplastic transformation of cells and during cancer progression and metastasis.

Online databases like Gene Expression Omnibus (GEO) and Array Express report increasing number of gene expression and microRNA expression data. The availability of public datasets has attracted the attention of bioinformaticians and articles on meta analysis are increasingly published. In this thesis, bioinformatics approaches to study the role of microRNAs in the regulation of Normal T cell development and pathogenesis are presented.

The work may be subdivided in two main parts:

Part 1 - bioinformatics analysis of genes and microRNAs in human T cell development on a novel set of data;

Part 2 - meta analysis of gene and microRNA expression data in T-ALL to reconstruct a TF-gene-microRNA network.

In the following sections, the main biological concepts and literature important for the development of the thesis are introduced.

1.1 T cell progenitors in the bone marrow

Bone marrow T cell progenitors in experimental murine models

Different kinds of blood cells (granulocytes, monocytes, erythrocytes, megakaryocytes, B and T cells originate from hematopoietic stem cells (HSCs), which express high levels of cell surface markers like CD117(kit) and Sca-1. There are several models which have been proposed to elucidate the mechanism of hematopoesis. According to Adolfsson et.al [1] specific population of these HSCs called short term haematopoetic stem cells develop into lymphoid primed multipotent progenitors (LMPP) (also known as ELP). These LMPPs have high levels of FLT3 and give rise to common lymphoid progenitor cells (CLPs) and Granulocyte-monocyte progenitors (GMPs). CLPs have high IL-7R alpha expression and are the true ancestors of B and T cells. These cells have very less potential to develop into myeloid lineage or megakaryocyte or erythroid lineage.

Development of T cell lineage from CLP is mainly dependent on Notch Signaling. Pui et.al [2]has shown that mice which were reconstituted with bone marrow transduced with retrovirus encoding a constitutively active form of Notch1, showed an early block in B cell lymphopoesis. However, in a very interesting observation, Heinzel et.al [3] has showed that T cell progenitors (termed TMP - thymic multipotent progenitors) suppress anti- T lineage potential only after arriving in the thymic microenvironment. The journey from bone marrow to the thymus (where T cells mature further) is dependent on expression of a gene called CCR9,

which helps in thymic homing of T cell progenitors. Schwarz et.al [4] has shown that Flt3+ signaling and concomitant CCR9 expression by common lymphoid progenitors is necessary for thymic homing of pre thymic cells. HSCs injected into the blood of unirradiated mice were unable to settle within the thymus, whereas downstream CLP rapidly generated T lineage cells following intravenous transfer [4].

In another paper Scimone et.al [5] has mentioned that P-selectin glycoprotein ligand 1 (PSGL-1), α4β1/vascular cell adhesion molecule (VCAM) 1, lymphocyte function-associated antigen 1/intercellular adhesion molecule (ICAM) 1, and CCL25/CCR9 are key components of the unique thymus-specific recruitment pathway. Therefore, it is very clear that bone marrow progenitors of T cells must acquire the potential to migrate to thymus where they undergo further development to become functional T cells. After leaving the bone marrow the T cell progenitors circulate in the blood for a very short time. As a result there are few circulating T cell progenitors in the blood. However it is interesting to note that a specific type of T cell progenitors called CTPs (Circulating T cell progenitors) are a pre thymic population of cells (phenotype: c-kit low Thy1+) found in blood of adult mouse, which possess a strong T cell potential (very low B and NK cell potential) and are not affected by inhibition of Notch signaling [6].

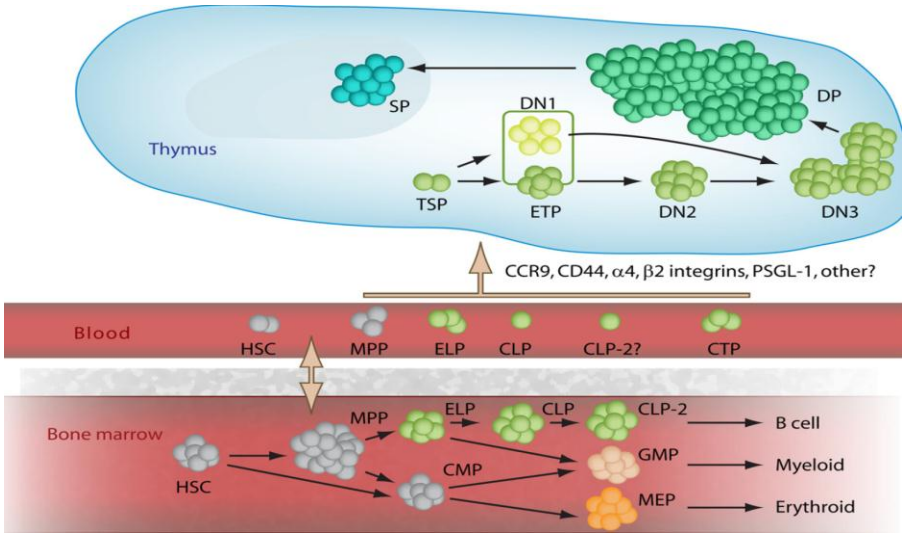

Figure 1.1 Progenitor-Successor Relationships in T Cell Development within the bone marrow, Blood and Thymus [7].

Human T cell progenitors in Bone marrow

It is very important to observe that the antigens expressed in murine haematopoesis are different from human haematopoesis although there are underlying similarities. In both the species mature functional cells develop from multipotent haematopoetic progenitors. Similar to murine models human bone marrow has CLPs, CMPs (common myeloid progenitors) and MEPs (megakaryocyte-erythrocyte progenitors).

Pioneering work by Markus.G.Manz et.al. [8] showed that lin-CD34+CD38+ human bone marrow cells which are IL-3R lo CD45RA- are common myeloid progenitors, where as IL-3R lo CD45RA+ cells are granulocyte monocyte progenitors. IL-3R-CD45RA- cells are megakaryocyte or erythrocyte progenitors. Another key paper on human haematopoesis by Anne Galy et.al [9] states that CD34+, CD38+ CD45RA+ human bone marrow cells, which co express CD10 has the potential to develop into T cells, Natural killer cells or dendritic cells and lack erythroid myeloid and megakarocyte lineage potential. Journey from bone marrow to

thymus begins as early as eighth week of gestation in human. There are two types of migration- Vasculature dependent and Vasculature independent. Vasculature in dependent migration starts very early and is dependent on chemo tactic action of two molecules CCL21 and CCL25 which are expressed in thymic primordium where as vasculature dependent migration starts at the later stages of embryogenesis and also postnataly.

1.2 T cell development in Thymus

T cell development in Thymus of mice

T cell progenitors enter the thymus in the cortico-medullary junction and migrate towards the subcapsular zone. During this journey they are in close contact with the thymic stromal cells which express delta like ligands for the Notch receptors. Therefore the Notch Signaling is extremely important for T cell differentiation in the thymus. In this context it is important to note that three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development [10].T cell development within the thymus is an extremely complex phenomenon with several independent research groups reporting different models for interthymic T cell development. CD3- CD8-CD44+ CD25- Kit hi population are the earliest population of T cell progenitor in the thymus and are variously termed Double-Negative 1 (DN1), early thymic progenitors (ETPs), or DN1a cells. It is important here to mention that some of the early thymic progenitors (ETPs) retain myeloid lineage potential [11] and therefore it is suggested they do not originate from common lymphoid progenitors (CLPs) but from lymphoid primed progenitor cells (LMPPs). DN1 develops to DN4 and the stages of development can be identified by the expression of

CD44+CD25+ (DN2), CD44-CD25+ (DN3) and CD44-CD25- (DN4). At DN2 stage multi lineage potential of thymocytes to develop into myeloid cells, NK cells or dendritic cells is completely lost. When the thymocytes arrive at the DN3 stage correct rearrangement of V(D)J genes occur, which allows the expression of TCR beta, TCR gamma and / or TCR delta genes. There are two types of thymocytes which are formed at this stage- one is alpha-beta receptor expressing thymocytes and another is gamma-delta receptor expressing thymocytes depending on the type of gene arrangement. Expression of TCR beta genes qualifies the thymocytes for "beta selection" which ultimately turns on CD4 and CD8 receptors on the cell surface. When the thymocytes, express both these receptors they have reached the double positive stage of thymocyte development.

The alpha beta receptors expressing double positive thymocytes undergo through three key events 1) Positive selection 2) Negative Selection 3) Lineage differentiation i,e they either become CD4 positive or CD8 positive. Thymocytes at this stage interact with self peptide bound MHC molecules (I and II) and those thymocytes which do not interact die. This event is called positive selection and is an ideal mechanism to eliminate non reactive thymocytes. After positive selection occurs, thymocytes which have receptors with very high avidity for self peptide bound MHC are eliminated by TCR induced programmed cell death. This process is called negative selection and is an essential program to eliminate those thymocytes which undergo through positive selection and may have potential for autoimmune reaction. Another important event that occur here is the lineage choice between CD4 or CD8 single positive stage. It is very interesting to observe that the functional immune system is designed in such a manner that it matches with MHC specificity. Those thymocytes which interact with self peptide bound MHC-II molecules become CD4 T helper cells and those which interact with MHC-I become CD8 cytotoxic T cells.

Genes and Transcription factors involved in Thymocyte Development in Mice

Transcription factors play stage specific roles in T cell development. The most important transcription factor which must be silenced at the DN2 stage for further T cell lineage progression is the ETS family transcription factor known as PU.1. Other transcription factors which has to be silenced before beta selection occurs are CEBP alpha, GATA2 and TAL1. All these transcription factors are responsible for pre thymic lineage plasticity. On the other hand there are some transcription factors which play essential role in T cell development, beta selection and survival of alpha-beta thymocytes. Wakabayashi et.al [12] stated that Bcl11b is essential for alpha-beta thymocyte survival.

Similarly, HEBalt transcription factor and GLI2 (involved in hedgehog signaling are upregulated in early double negative stages and are important for T cell lineage differentiation. Between DN2 and DN3 transition several transcription factors like ETS1, ETS2, LEF1 are upregulated. DN3 also has a high level of transcription factor SPIB and Deltex1 (Notch target gene) but they are turned off after beta selection. SOX13 transcription factor decides between alpha beta and gamma-delta lineage thymocytes [13].

It is very interesting to note that in response to Notch signaling there are a restrictive set of hematopoietic precursors which starts expressing T cell specific genes like GATA3 and TCF1 transcription factors. There are four transcription factors which have been identified to "initiate" T cell lineage - these are Ikaros, PU.1, RUNX1 and CBF beta. On the other hand, a negative regulator of T cell lineage called LRF (leukaemia/lymphoma related factor, also known as Zbtb7a or Pokémon) counteract with Notch3 and Notch1 to keep the development of T cell lineage under control [14].

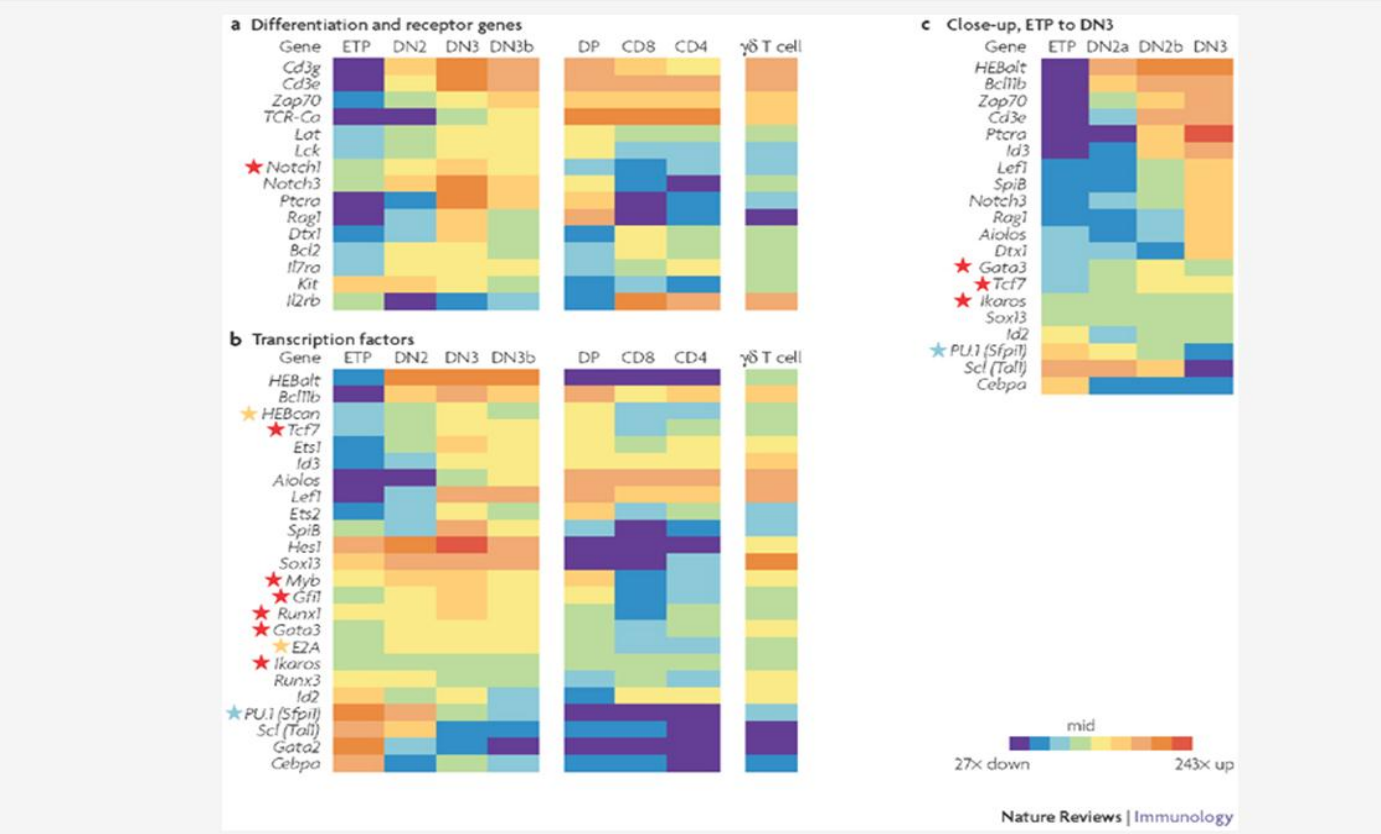

Figure 1.2 Expression levels of differentiation and regulatory genes in early stages of T cell development [15].

Transcription factors and genes also play a major role in development of Double positive alpha beta thymocytes into single positive (CD4 or CD8). During positive selection T cells which cannot initiate TCR signaling die of apoptosis. Anti-apoptotic genes like Mcl1 (a Bcl-2 family member) plays key role in T cell survival after its expression is upregulated by TCR signaling [16].

As mentioned earlier,E2A and HEB transcription factors have key roles to keep the cell at the DP stage and prevent further differentiation until the beta selection gets over. Therefore, these are the main transcription factors which must be switched off by the cell if it wants to get differentiated into single positive from double positive stage [17]. It is again important to

mention in this context that four mature T cell genes Il7r, foxo1, Ccr7 and klf2 are negatively regulated by these transcription factors (E2A and HEB). Apart from these, pioneering work by Patrik.S.Costello [18] clearly indicated that during positive selection ERK pathway component Elk4 triggers Egr1 which increases the level of Id3, a key repressor of E2A-HEB transcription factors. In summary, it is very clear that several transcription factors are involved in DP to SP transition in mouse thymocytes.

The two main transcription factors which are involved in deciding the final stage of T cell differentiation in the thymus into SPCD4 or SPCD8 are Runx3 and Thpok. Setoguchi et.al [19] showed that DP thymocytes differentiate into CD4 SP in presence of transcription factor Thpok. They also demonstrated that a Runx binding sequence in the locus of Thpok, silences its action and thus prevents CD4 lineage choice of the cell. Wang.L et.al [20] showed that GATA3 transcription factor binds to Thpok and promotes its expression. Sakaguchi et.al [21]further demonstrated that a transcription factor named MAZR binds to the silencer of the gene encoding ThPok and therefore takes part in CD8 SP lineage choice of the thymocytes. After the final differentiation of the thymocytes inside the thymus, they egress the thymus to reach the peripheral lymphoid organs. In this process, the major role is played by zinc finger transcription factor klf2, which is upregulated by foxo1. According to Kerdiles et.al [22] FOXO1 is an important transcription factor involved in T cell trafficking, homing and survival as it regulates IL7R,CCR7 and P-selectin molecule.

Therefore, in summary multiple transcription factors and genes work in tandem to regulate thymocyte development inside mouse thymus.

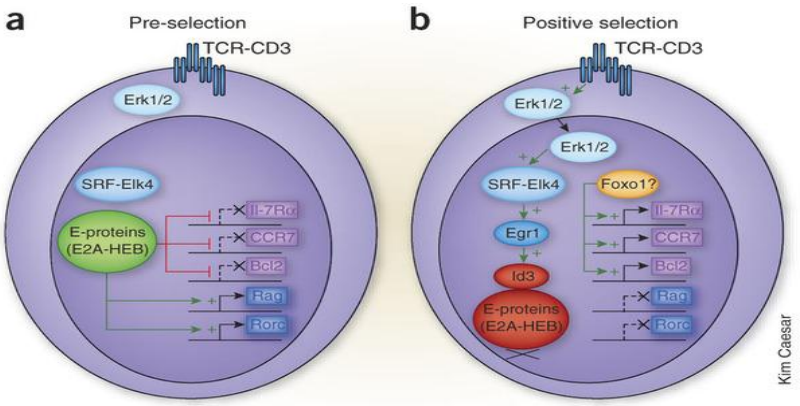

Figure 1.3 Transcriptional circuitries in differentiating αβ T cells [15]: a. Pre-selection; b. Positive Selection.

Thymocyte Development in Human Thymus

For obvious reasons T cell development is mostly studied in mouse [23]. The only paper from which we get comprehensive information about human T cell development is from the paper of W.A Dik et.al [23]. They isolated different subsets of human thymocytes based on surface markers and performed Gene expression profiling and PCR analysis in order to understand the human T cell receptor gene recombination and corresponding gene expression in great detail. In human, three distinct DN stages can be recognized- $CD34^{+}CD38^{-}CD1a^{-}$ stage that represents the most immature thymic subset and the consecutive $CD34^{+}CD38^{+}CD1a^{-}$ and $CD34^{+}CD38^{+}CD1a^{+}$ stages. Human DN thymocytes mature via an immature single positive (ISP $CD4^{+}$) and a DP stage into $CD4^{+}$ or $CD8^{+}$ SP T cells that express functional T cell receptors (TCR) and that exit the thymus. Based on hierarchical clustering analysis of different subsets of thymocytes and expression profiles of three key differentiation genes RAG1, RAG2 and PTCRA [23] Dik et.al [23]suggested that human T cell development has many similarities with mouse T cell development in the thymus.

1.3 Identification of key oncogenes and Transcription factors in T- cell Acute lymphoblastic leukemia

Approximately 15% and 25% of the newly diagnosed cases of acute lymphoblastic leukaemia (ALL) in children and adults, respectively, are T-cell ALL (T-ALL) and are historically linked with a poor prognosis [24].

Malignant thymocytes arise from normal counterparts during intrathymic T cell development and some of the oncogenes have been associated with differentiation arrest at particular stages of thymocyte development. The main cytogenetic features of the malignant thymocytes are chromosomal translocations between strong promoter elements of T cell receptor genes (TCRA,TCRG,TCRB) present in chromosome 7 and 14 and oncogenic transcription factors like TAL1, LYL1 and HOX11. It is also interesting to note that some of these transcription factors and oncogenes are present in very low level in T cells when compared to their malignant counterpart. Another interesting hallmark of T cell transformation are the phenomena of activating mutations in genes like NOTCH1 and FBW7. In this context, it is also important to state that the four proteins of NOTCH family - NOTCH1, NOTCH2, NOTCH3 and NOTCH4 have significant roles in T cell development as well as T- ALL induction [24]. Therefore, it is clear from the above discussion that a combined role of oncogenic transcription factors and activating mutations drive the development of T cell Acute lymphoblastic leukemia. Some of the transcription factor families involved in T-ALL are discussed below.

Basic helix-loop-helix (bHLH) transcription factors

One of the major bHLH transcription factor which is activated in 23% of T-ALL is TAL1. TAL1 forms heterodimers with other bHLH proteins like HEB and E2A and acts as a transcriptional

repressor. Teresa Palomero [25] has proposed a complex transcriptional network for TAL1 in which TAL1 not only acts a transcriptional repressor but also as a transcriptional activator which disrupts normal T cell homeostasis. Another bHLH protein which has structural motif similarity with TAL1 is LYL1. LYL1 is not expressed in normal thymocytes and is expected to play major roles in development of several blood tumours.

HOX family transcription factors

HOX family of transcription factors encoded by homeobox genes play significant role in T-ALL development. Translocations involving T cell receptor regulatory regions and HOX11 gene makes it highly activated in many cases of T cell lymphoblastic leukemia. HOX11 which is otherwise not expressed in normal thymocytes, is expressed aberrantly in T-ALL causing a developmental block at the DP stage of thymocyte differentiation.

Notch Signaling and Notch1 Transcription factor

Translocation involving NOTCH1 and T cell receptor locus regulatory regions is a rare phenomenon in T-ALL but an activating mutation of HD domain or PEST domain of NOTCH1 protein makes it stable by masking it from the effect of ubiquitin ligase. As a result the notch receptor intracellular domain becomes activated and generates a signaling pathway, up regulating a plethora of target genes. The key target genes of this pathway are MYC and NFKB which further alter the normal T cell development and promote leukemogenesis. An important point to note in this regard is that 40% of genes identified by ChIP-chip as targets of NOTCH1 are involved in cell metabolism and protein biosynthesis. Two major pathways that are altered by NOTCH signaling are PI3-AKT signaling pathway and NFAT signaling pathway. Notch3, another member of the Notch family may be involved in T cell leukemogenesis.

Bellavia, D et.al [26] generated transgenic mice in which intracellular Notch3 was overexpressed specifically in T cells. These mice developed multi-organ infiltration of T lymphoblasts. Therefore, similar to NOTCH1, NOTCH3 may play role in T cell leukemogenesis.

Translocation	Involved gene	Fusion gene function	Frequency
t(7;10)(q34;q24) and t(10;14)(q24;q11)	*TLX1 (HOX11)*	Transcription factor	7% children 31% adults
t(5;14)(q35;q32)	*TLX3 (HOX11L2)*	Transcription factor	20% children 13% adults
inv(7)(p15q34), t(7;7)	*HOXA* genes	Transcription factor	5%
t(1;14)(p32;q11) and t(1;7)(p32;q34)	*TAL1*	Transcription factor	3%
t(7;9)(q34;q32)	*TAL2*	Transcription factor	<1%
t(7;19)(q34;p13)	*LYL1*	Transcription factor	<1%
t(14;21)(q11.2;q22)	*BHLHB1*	Transcription factor	<1%
t(11;14)(p15;q11)	*LMO1*	Protein-protein interaction	2%
t(11;14)(p13;q11) and t(7;11)(q35;p13)	*LMO2*	Protein-protein interaction	3%
t(1;7)(p34;q34)	*LCK*	Signal transduction	<1%
t (7;9)(q34;q34.3)	*NOTCH1*	Fate determination, differentiation	<1%
t(7;12)(q34;p13) and t(12;14)(p13;q11)	*CCND2*	Cell cycle activator	<1%

Figure 1.4 Rearrangements involving T cell Receptors Gene in T-ALL [27].

1.4 Selected Gene Expression profiles in T-ALL

AUTHORS	GENE EXPRESSION PROFILES
Ferrando A.A.(2002)	Using oligonucleotide microarrays, several gene expression signatures that were indicative of leukemic arrest at specific stages of normal thymocyte development were identified: *LYL1*$^+$ signature (pro-T), *HOX11*$^+$ (early cortical thymocyte), and *TAL1*$^+$ (late cortical thymocyte)
Yeoh E.J. (2002)	Oligonucleotide microarrays were used to analyze the pattern of genes expressed in leukemic blasts from 360 pediatric ALL patients. Distinct expression profiles identified each of the prognostically important leukemia subtypes, including T-ALL, *E2A-PBX1*, *BCR-ABL*, *TEL-AML1*, *MLL*rearrangement
Ferrando A.A.(2003)	Interesting article about distinct subtype of T cell lineage leukemia "T-ALL-MLL" which arises from T cells expressing gamma-delta T cell receptors(LYL1+)
Chiaretti S. (2004)	Identification of 19 genes which were differentially expressed between patients who had complete remission (CR) and patients who had subsequent relapse from T-ALL
Kohlmann A.(2004)	Gene expression patterns of the cohort of paediatric leukemia patients (including T-ALL) from Eng-Juh Yeoh et.al was collected and applied to a patient cohort of adult leukemia to see if that signature can differentiate in a similar manner also adult leukemias
Martinez-Delgado B. (2004)	Gene Expression Profiles to differentiate between Peripheral and Lymphoblastic Lymphomas
Hoffmann K. (2004)	Gene expression profiling t was done to find out the target genes of HOX11, the most important deregulated genes in T-ALL
Palomero T. (2006)	Gene Expression profiling data of primary human T-ALL subjects were used to identify direct targets of TAL1 and indentify a transcriptional network downstream of TAL1/SCL
Clappier E.(2007)	Identification of TCRB-MYB chromosomal translocation in very young T-ALL patients which has a characteristic gene expression signature quite different from other T-ALL subtypes
Coustan-Smith E. (2009)	Gene expression profiling datasets of T-ALL was used and postulated that T-ALL originating from early T-cell precursors (ETPs), a recently defined subset of thymocytes that retain stem-cell-like features, would respond poorly to lymphoid-cell-directed therapy.

Sanda T. (2010)	Gene set enrichment analysis (GSEA) and connectivity map analysis (CMAP), using gene expression data from both mouse and human T-ALL cells were used to identify changes in expression profiles during distinct phases of *NOTCH1*-mediated T-ALL development and to gain insights into small-molecule inhibitors that would synergize with or substitute for GSIs in the disease
De Keersmaecker K. (2010)	A gene expression profiling was done to identify gene signature corresponding to TLX1-transgenic mouse and compared it to human T-ALL samples.
Cleaver A. (2010)	Gene expression profiling from T-ALL patients were used to identify a five gene classifier which can accurately predict clinical outcome in T-ALL patients.
Messina M. (2010)	Gene expression profiling was done to identify distinct set of genes which encode protein tyrosine kinase and which gets differentially regulated in T-ALL subsets.
Haferlach T. (2010)	Massive study to understand the clinical utility of microarrays based gene expression profiling in the diagnosis and subclassification of leukemia.

Table 1.1 Selected gene expression profiles in T-ALL from literature.

1.5 MicroRNAs in the Immune System

1) A General overview of MicroRNA Biology

MicroRNAs are small nucleotide molecules (approximately 22- 23) which are non coding in nature and which bind to mRNA molecules and cause degradation or repress their translation [28]. First microRNA, lin-4 was discovered in *C.Elegans* and was shown to be involved in larval developmental by reducing the level of LIN-14 protein [29]. Approximately 30% of all protein coding genes are predicted to be under the control of microRNAs. Therefore, genes are not only regulated during transcription by multiple transcription factors and co factors but also by microRNAs, which binds to these genes post transcriptionally and repress the

formation of the protein. MicroRNA precursors are transcribed as several nucleotides length molecules and are processed inside the nucleus by DROSHA-DGCR8 microprocessor complex to form pre-miRNAs (approximately 70 nucleotides of hairpin structure) [30].These pre-miRNAs are exported outside the nucleus by a Ran-GTP complex called Exportin-5 [31]. Inside the cytoplasm, these pre-miRNAs are further processed by DICER to form approximately 22-23 nucleotide length double stranded RNA molecules.

These double stranded RNA molecules are recognized by RISC complex and one of the strand (guiding strand) binds to the mRNA causing its degradation or translational repression. The other strand called passenger strand gets degraded. In this context it is interesting to discuss two papers - one by Biasiolo et.al [32] and another by Ro.S et.al [33] which describe that the passenger strand is not always degraded (also known as star microRNA) but are concurrently or alternatively expressed with the other strand depending on the cell type.

2) MicroRNAs in innate immune response - Regulation of key genes and transcription factors

a) MicroRNAs in myeloid cell development

MicroRNAs are involved in both innate and adaptive immune system function. But since granulocytes, macrophages, dendritic cells and natural killer cells (components of innate immune response) form the first line of defence against the foreign antigens, role of microRNAs in regulating this branch of immune system is discussed first. From the developmental point of view, three microRNAs namely microRNA106a, microRNA17-5p and microRNA20a are involved in suppressing the transcription factor RUNX1, and thus

preventing differentiation of GMP into monocytes. During differentiation of monocytes into macrophages, above mentioned microRNAs are down regulated [34].

Another important master regulator transcription factor PU.1 upregulates microRNA424 which in turn down regulates another transcription factor NFIA and promotes monocytes differentiation [35]. Influence of microRNAs on granulocyte development from GMP is regulated by transcription factor Gfi1 which regulates microRNA21 and microRNA196b to control granulopoiesis [36]. Another very interesting paper on neutrophil differentiation and activation was reported by JB. Johnidis [37].

He showed that microRNA223 negatively regulates granulocyte proliferation and activation by targeting transcription factor Mef2c. It is also very important to note that microRNA223 can be induced by two transcription factors C/EBP-alpha and PU.1 and therefore these transcription factors play significant role in granulocyte biology.

b) MicroRNAs in inflammatory pathways

Five most important microRNAs involved in inflammatory responses of the macrophages are microRNA146, microRNA155, microRNA147, microRNA21, microRNA9. MicroRNA-146a is involved in negatively regulating NFKB mediated Toll like receptor (TLR) pathway, in response to various pro-inflammatory signals. It binds to the 3 prime UTR and down regulates two molecules IRAK1/2 or TRAF6 which are key adaptor molecules involved in TLR pathway. Therefore, it fine tunes the innate immune response by providing a negative feedback in response to inflammatory signals. This finding was first reported by Konstantin. D. Taganov et al [38], who performed a microarray profiling of 200 microRNAs in human monocytes and identified hsa-miR-155 and hsa-miR-132 other than hsa-miR-146a to be involved in innate immune response and pro-inflammatory pathways. Recently, hsa-miR-146a has been

projected as a major therapeutic and prognostic target in rheumatoid arthritis and osteoarthritis. MicroRNA-155, another important molecule involved in innate immune response as mentioned above is an effective negative modulator of TLR pathway in response to microbial stimuli. This function was reported by Maurizio Ceppi et.al [39] who also performed a microRNA profiling of human monocyte derived dendritic cells stimulated by lipopolysaccaride. MicroRNA21 role in repressing the gene PDCD4 was discovered by F.J.Sheedy [40]. By repressing PDCD4, microRNA plays an anti-inflammatory role by negatively regulating NFKB and IL-10 production after lipopolysaccaride stimulation. Flavia Bazzoni et.al [41] reported that MicroRNA9 targets NFKB1 transcription factor in response to lipopolysaccaride stimulated TLR4 signal and like microRNA-146 forms a negative feedback loop in response to pro inflammatory signal. Therefore, in summary different microRNAs play crucial role in fine tuning the innate immune system in response to microbial infections.

3) MicroRNAs in adaptive immune response - B cell related microRNAs and transcription factor.

Though adaptive immunity consists of both B and T cells response, in this section the role of microRNAs in B cell development is briefly discussed based on few published papers. A much detailed discussion of microRNAs in T cell development will be done in the subsequent sections of the introduction.

Similar to T cells, B cells also undergo through several steps of maturation from haematopoetic stem cells to mature B cells. During this process rearrangement / recombination of immunoglobulin genes (*Variable Diverse Joining*) occur in the bone marrow. (instead in T cells, T cell receptor genes Variable Diverse Joining occur in thymus). During

this process of maturation, microRNA150 targets c-myb transcription factor [42], a key transcription factor involved in B cell development. Again similar to T cells, B cells react with antigens in the peripheral lymph nodes where they undergo germinal centre reaction and form either plasma cell (antibody producers) or memory B cells. Basso.K et.al [43] reported distinct microRNA signatures corresponding to specific stages of B cell development in the periphery (naive, centroblast, memory) by performing microRNA profiling of these cell types. Another microRNA which has a reported role in B cell germinal centre reaction is microRNA155. It is also reported to play key role in class switching mechanism of antibody subtypes in peripheral plasma B cell [44]. Therefore in summary, microRNAs also play significant role in the maturation and differentiation of B cells in bone marrow and peripheral lymphoid organs.

1.6 MicroRNAs in T cell Development

Involvement of microRNAs in T cell development inside the thymus and also in T cell activation and differentiation in the periphery is studied in great detail by immunologists all over the world. Different subpopulations of T cells play diverse role in adaptive immune response against various diseases. As for example, different T cell populations not only infiltrate growing tumors in the body but also play key roles in combating against many deadly virus, which specifically infect certain sub population of T cells.

Therefore, studying the role of microRNAs in normal T cell physiology and also in T cell pathologies may provide the researchers with interesting clues regarding the understanding of various diseases and neoplasms. In the following paragraphs, brief descriptions of different microRNA profiling reports on Normal T cell subpopulations are described.

1) Microarray profiling of the murine haematopoetic system by Silvia Monticelli et.al, [45]

It is one of the earliest works on microRNA profiling of the haematopoetic system. They performed microRNA profiling with 181 mature miRNAs. Even though only selected hematopoietic cell lineages were analyzed, each differentiation step was characterized by changes in miRNA expression, with some miRNAs showing increased and some showing decreased expression. MiR-150 expression is of particular interest: this miRNA is up-regulated during the developmental stages of B and T cell maturation, but down-regulated again during the further differentiation of naïve T cells into effector Th1 and Th2 cells.

2) Microarray profiling of murine Naive, Effector and Memory CD8 T cells by Haoquan Wu et.al, [46]

In this paper authors have used three different technologies to profile microRNAs in different subpopulations of CD8 T cells- miRNA Microarray, Real Time PCR and small RNA cloning. They reported that 7 miRNAs (miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-are highly expressed in CD8 T cells as compared to other microRNAs.)

3) Differential expression of microRNAs in human hematopoietic cell lineages by Michaela Merkerova et al

This interesting paper [47] describes the microRNAs which are upregulated specific to a haematological subpopulation. (isolated from peripheral blood of 10 healthy individuals)

4) A mammalian microRNA expression atlas based on small RNA library sequencing [48]

In this interesting paper considered as one of the milestone papers in microRNA research authors sequenced small RNAs in haematopoetic sub populations.

5) Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in $CD4^+$ T cells by the microRNA miR-125b [49]

In this landmark paper authors have isolated 17 different subpopulations of human peripheral CD4 T cells and performed microRNA profiling. From this expression profiling they identified microRNA-125b as the most differentially regulated microRNA between naive and effector memory CD4 T cells. In order to identify the targets of hsa-miR-125b, they combined gene expression analysis with the predicted targets of hsa-miR-125b. They have termed this method as ACEPT (anti-correlated expressed predicted targets). 72 ACEPT mRNAs for miR-125b were significantly enriched for the biological function of cell-mediated immune response and the canonical pathway of helper T cell differentiation.Furthermore, ACEPT mRNAs for miR-125b generated a network of 133 gene products that included several molecules involved in T cell differentiation, such as cytokines, cytokine receptors and transcription regulators (Rossi,RL, Nature Immunology, 2011). From this list of ACEPT genes they chose 14 genes involved in peripheral CD4 T cell differentiation. Out of these 14 genes they reported 4 genes IFNG, IL2RB, IL10RA and PRDM1 to be direct targets of hsa-miR-125b.

6) MicroRNAs in T cell development and differentiation- Regulation of key genes and transcription factors

Before discussing the role of microRNAs in T cell development and differentiation, it will be interesting to mention the paper by Sandberg.R et.al [50] which tells that prolifirating T cells have transcripts whose UTRs are shorter than resting T cells and as a result have much more fewer microRNA targets than resting T cells. However, there are some interesting experimentally validated targets in T cells. Li Q,J. et.al [51] identified that microRNA181a represses signaling molecules of MAP kinase pathways namely,

DUSP5 and DUSP6, SHP-2 and PTPN22 and inhibition of these microRNA can result in marked decrease of positive and negative selection in mature T cells of mouse. In another paper by Joel.R.Nielson et.al [52], it was shown that several genes, CD69, Bcl-2,Tox,transcription factor Runx1, and transcription factor Egr1 are targeted by microRNA181. Interesting paper by Changchun Xiao et.al [53] showed that microRNA cluster 17-92 is highly expressed in immature T cells and its expression gradually decreases in mature T cells. They also demonstrated that this cluster regulates Pten and Bim**,** two genes involved in apoptosis and cell proliferation pathways. In another landmark paper, Curtale, G et.al [54] demonstrated that hsa-miR-146a is not only involved in innate immune response but also is an active player in adaptive immune response. They showed that microRNA-146a is highly expressed in activated human T cells and has an antiapoptotic function. It also targets FADD**,** a gene involved in apoptotic pathway. Finally it is time to mention about a paper by Li-Fan-Lu et.al [55] where he found that microRNA155 induced by transcription factor FOXP3 plays crucial role in a specific sub- population of T cells called Tregs in which it suprreses SOCS1 and impairs the function of transcription factor STAT5. In conclusion, it can be said that microRNAs play significant role in different subpopulation of T cells by regulating several genes and transcription factors involved in multiple cellular pathways.

1.7 MicroRNAs in T cell acute lymphoblastic leukemia

After writing about the role of microRNAs in normal T cells, it is necessary to understand the roles of microRNAs in one of the T cell related neoplams, T cell acute lymphoblastic leukemia (T-ALL). For this purpose a thorough literature search was performed to collect the papers which deal with microRNAs associated with T-ALL.

The first microRNA profiling on acute lymphoblastic leukemia (ALL, in which T-ALL samples were also present) was published by ML den Boer group in the year 2009 [56].

The role of transcription factors TLX1, TLX3, NKX2-5 (associated with translocation: T cell receptor promoter elements) in increasing the levels of microRNA17-92 cluster (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92) was reported by Stefan Nagel et.al [57]. Kaddar.T et.al [58] published a paper in which they reported the prognostic value of microRNA16 in T cell acute lymphoblastic leukemia. Valerio Fulci et.al [59] performed microRNA profiling on both B and T lineage lymphoblastic leukemia and identified three microRNAs miR-148, miR-151, and miR-424 as discriminative of T-lineage versus B-lineage ALL. Pioneering works on microRNA research of T-ALL was performed by Konstantinos J Mavrakis et.al [60]. They identified microRNA 17-92 cluster (microRNA19) targets tumor suppressors Bcl2l11, Prkaa1, Pten and PP2A and concluded that microRNA 19 is highly expressed in T-ALL and is oncogenic in nature. In the other paper, they identified five microRNAs namely microRNA19b, microRNA20a, microRNA26a, microRNA92, microRNA223 as highly expressed microRNAs in T-ALL and discovered that they target (overlapping targets) tumor suppressor genes like PTEN, BIM, PHF6, NF1 and FBXW7 to form a cooperative network. Before writing about few other recent papers and microRNA profiling experiments, it is necessary to mention that other than microRNA 17-92 cluster

another microRNA which is slowly gaining importance in T-ALL microRNA research is microRNA 223. Although, microRNA223 has been marked as a myeloid specific microRNA and has been studied widely, it is also highly expressed in T-ALL cases [60].

Further, in a very exciting paper, Sabina Chiaretti et.al [61] has studied 69 untreated T-ALL cases by Real-Time Quantitative PCR and reported high expression of microRNA-223 and myeloid specific genes like CEBPA, MPO, GRN and IL8.

In another very recent paper, Samuel Gusskott et.al [62] reported that microRNA-223 is highly upregulated (identified by microRNA profiling) in T-ALL cells (Jurkat and P12 Ichikawa cell line) while they were treated with GSI to block Notch Signaling. They also stated that Notch pathway regulated microRNA223 may directly repress target gene IGF1R, one of the main genes in IGF1 signaling pathway (which is also regulated by Notch pathway).

2. AIM OF THE STUDY

The main aim of the study is to investigate the role of microRNAs in the regulation of normal T cell development and pathogenesis. In order to achieve this aim, a bioinformatics analysis was performed on normal thymocytes dataset and a meta analysis was performed on T cell acute lymphoblastic leukemia dataset.

AIM1

Analyze the microRNA and mRNA expression profiles corresponding to Double Positive and Single Positive stages of thymocyte development and identify microRNAs regulating important genes during thymocyte development.

AIM2

Compare the gene expression data of normal thymocytes and T-ALL and combine this data with microRNAs differentially expressed to construct a network of microRNAs targeting differentially regulated genes and transcription factors.

3. METHODS

PART 1 - Bioinformatics analysis of genes and microRNAs in human T cell development

3.1 Data

MicroRNA expression profiles in normal human thymus tissue, purified DP, SP $CD4^+$, and SP $CD8^+$ human thymocytes were investigated with the Human miRNA Microarray kit Version 2.0 (Agilent Technologies), which allows detection of 723 known human and 76 human viral miRNAs (miRBase Version 10.1.). Six biologic replicates for each T-cell population were hybridized on the arrays. Total RNA was labeled with the miRNA Complete Labeling and Hyb Kit (Agilent Technologies); fluorescent signals were extracted and analyzed with Feature Extraction Software (Version 9.5.3.1; Agilent Technologies).

Gene expression profiles of DP and SP $CD4^+$ human thymocytes were obtained by hybridization on Human genome U-133 PLUS microarrays (Affymetrix). Three to 6 biologic replicates for each sample were hybridized on the arrays. Gene expression data were extracted by GeneChip software Affymetrix Microarray Suite 5.0 [MAS 5.0].

3.2 Computational analysis

Computational analysis was performed by the candidate in collaboration with Alberto Corradin. Gene-Microarrays were normalized by applying the Robust Multichip Average (RMA) procedure, whereas no normalization method was applied in case of microRNA microarrays. Differentially expressed features were identified by Significance Analysis of Microarrays (SAM) [63], as implemented in the BRB Array Tools <http://linus.nci.nih.gov/BRB-

ArrayTools.html>, ver 3.6, and adopting a False Discovery Rate (FDR) of 0.001. Hierarchical clustering of samples was performed using the same software package. Heat maps were obtained with Genesis software package. Functional Annotation Clustering was performed using DAVID software <www.david.abcc.ncifcrf.gov> [64].

PART 2 – Meta analysis of gene and microRNA expression data in T-ALL

3.3 Genes Differentially Expressed between T-ALL and Normal Thymocytes

A data set containing 174 T-ALL samples [65] was collected from Gene Expression Ominibus <http://www.ncbi.nlm.nih.gov/geo/> (GSE 13204) and compared to 6 normal thymocytes samples, analysed in the first part of the thesis. Data were normalized using Robust Multichip Average (RMA) method and genes differentially regulated between normal thymocytes and T-ALL were evaluated using the limma package [66] in collaboration with Angela Grassi.

3.4 TFs Differentially Expressed between T-ALL and Normal Thymocytes

Among the differentially expressed genes, transcription factors highly up regulated (> 2.5 Fold Change) were selected using the recently developed HTRIdb database (2012). Next a

software called CONFAC [67] was used to look for the binding sites of these transcription factors in strongly down and up regulated genes (Fold change < -5 or > 5). Conserved transcription factor binding site (CONFAC) software enables the high-throughput identification of conserved transcription factor binding sites (TFBSs) in the regulatory regions of hundreds of genes at a time. The output of the CONFAC software is a table in which each column represents a given gene, and each row represents a transcription factor in the TRANSFAC database [67].

3.5 MicroRNAs Differentially Expressed between T-ALL and Normal Thymocytes

MicroRNAs have emerged as key players in the regulation of immune responses, both innate and adaptive. Since microRNAs are known to regulate genes, differentially expressed microRNAs in T-ALL vs normal thymocytes were collected from literature in order to hypothesize relationships between genes, transcription factors and microRNAs, all differentially regulated between T-ALL and thymocytes. MicroRNA data were obtained from [68] and are reported in Table 3.1.

MicroRNA	FC	STATUS
hsa-miR-107	5.66	Upregulated
hsa-miR-126	5.89	Upregulated
hsa-miR-145	14.73	Upregulated
hsa-miR-190	2.64	Upregulated
hsa-miR-223	8.71	Upregulated
hsa-miR-27a	2.37	Upregulated
hsa-miR-320a	2.05	Upregulated
hsa-miR-132	0.008	Downregulated
hsa-miR-146a	0.19	Downregulated
hsa-miR-151-3p	0.07	Downregulated
hsa-miR-193b	0.07	Downregulated
hsa-miR-205	0.001	Downregulated
hsa-miR-206	0.005	Downregulated
hsa-miR-22	0.31	Downregulated
hsa-miR-26a	0.45	Downregulated
hsa-miR-296-5p	0.06	Downregulated
hsa-miR-433	0.002	Downregulated
hsa-miR-549	0.07	Downregulated
hsa-miR-564	0.01	Downregulated
hsa-miR-566	0.03	Downregulated
hsa-miR-575	0.01	Downregulated
hsa-miR-601	0.14	Downregulated
hsa-miR-639	0.02	Downregulated
hsa-miR-646	0.07	Downregulated
hsa-miR-650	0.13	Downregulated
hsa-miR-708	0.03	Downregulated

Table 3.1 Differentially Regulated microRNAs between T-ALL and Normal thymocytes.

MicroRNAs target prediction strategy

Predictions of microRNA targets in animals is a challenging task because of incomplete complementarily between microRNA and its target sites and lack of sufficient scientific knowledge about the process controlling the microRNA-mRNA interaction. Several microRNA target prediction algorithms have been designed to predict microRNA targets in animals.

In the paper of Baek et.al [69] it is mentioned that TargetScan and Pictar performed better than other considered softwares in identifying true targets for hsa-miR-223 (insilico targets for hsa-miR-223 were compared with down regulated proteins, identified by mass- spectrometric method). Also in 2009's paper of Papodopulus [70] it was indicated that both Pictar and TargetScan performed very well in terms of precision and sensitivity as compared to other softwares, when their predictions were compared with the pSILAC data of Selbach *et al* [71]. In another paper Shirdel et.al [72] have shown Pictar and TargetScan to have high Precision and PITA to have a high Recall, when compared to publicly available high-throughput microarray data. According to T.M. Witkos et.al, [73] the most suitable approach for miRNA target prediction seems to be dependent on the nature of the planned experiments. Also in the paper "MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression" [74] authors have used three software (PicTar, TargetScan, PITA) for their analysis. Considering all these above mentioned factors, it was decided to use an union of TargetScan(conserved) and PicTar(4) and intersect them with PITA ALL predictions (targets were downloaded from mirDIP database). Finally the results from the above three databases were merged with miRTarBase, a database of experimentally validated targets**.**

This was done to achieve 2 main purpose:

1) A balance was achieved between two main target recognition properties of softwares - a) Evolutionary Conservation and b) Target Site accesibility and also between two main measures of relevance a) High Recall (PITA ALL Targets) b) High Precision (TargetScan and PicTar).

2) A mutual intersection of all softwares used in mirDIP, showed that "PITA All Targets" shares about 85% of targets with both TargetScan (conserved) and PicTar(4), Figure 3.1, and so by intersecting the predicted targets of TargetScan union PicTar with PITA, a good number of reliable putative targets were obtained.

	Dianaresult	Tscannonconsvresult	Tscanconsvresult	pictar4	pictar5	RNA22	Pita	mirbase	mircrornaorg
Dianaresult	1	0.557246205	0.106884489	0.092857385	0.0330469	0.1952586	0.7377985	0.1149	0.239343817
Tscannonconsvresult		1	0.03941805	0.048511025	0.01145715	0.1804046	0.8460105	0.1166629	0.24128211
Tscanconsvresult			1	0.382557517	0.18164794	0.2279294	0.8785447	0.1581059	0.56928839
pictar4				1	0.3526245	0.2516824	0.8549798	0.1618439	0.501345895
pictar5					1	0.2631077	0.8531935	0.1620591	0.585319352
RNA22						1	0.341418	0.0497863	0.100704971
Pita							1	0.0765988	0.164708345
mirbase								1	0.250452261
mircrornaorg									1

Figure 3.1 Mutual Intersection of all softwares used in mirDIP. Normalized values are obtained by dividing the number of targets predicted by each software in the column by the number of targets predicted by the software in a row.

3.6 Integration of genes and microRNAs differentially expressed in T-ALL and Normal Thymocytes

The genes found to be targets of differentially regulated microRNAs were intersected with differentially regulated genes between T-ALL and Normal thymocytes in order to reconstruct a microRNA gene network. A further step was performed to reduce the number of genes in the network. Genes which were differentially regulated (>5 or < -5 fold) as well as putative targets of at least one of the 9 transcription factors were intersected with differentially regulated microRNA targets. A comprehensive gene -microRNA network was created with these microRNA- gene pairs along with the transcription factors by using "Advanced Network Merge Plugin in Cytoscape".

4. RESULTS

PART 1 - Bioinformatics analysis of genes and microRNAs in human T cell development

4.1 Differentially expressed microRNAs between Double positive and Single Positive thymocytes sub- populations

Hierarchical clustering indicated that each thymic population was characterized by a distinct miR expression pattern which reflected the developmental relationships across maturation stages in T precursors. In particular, DP thymocytes showed a miR profile quite distinct from those of CD4+ and CD8+ SP cells; on the other hand, expression profiles of the two SP populations showed important similarities (Figure 4.1).Significance Analysis of Microarray (SAM) revealed that 70 miRs were differentially regulated in SP compared to DP thymocytes (60 up-regulated and 10 down-regulated) (Figure 4.2). Interestingly, a general up-regulation was observed in the maturation of thymocytes from the DP to the SP stage.

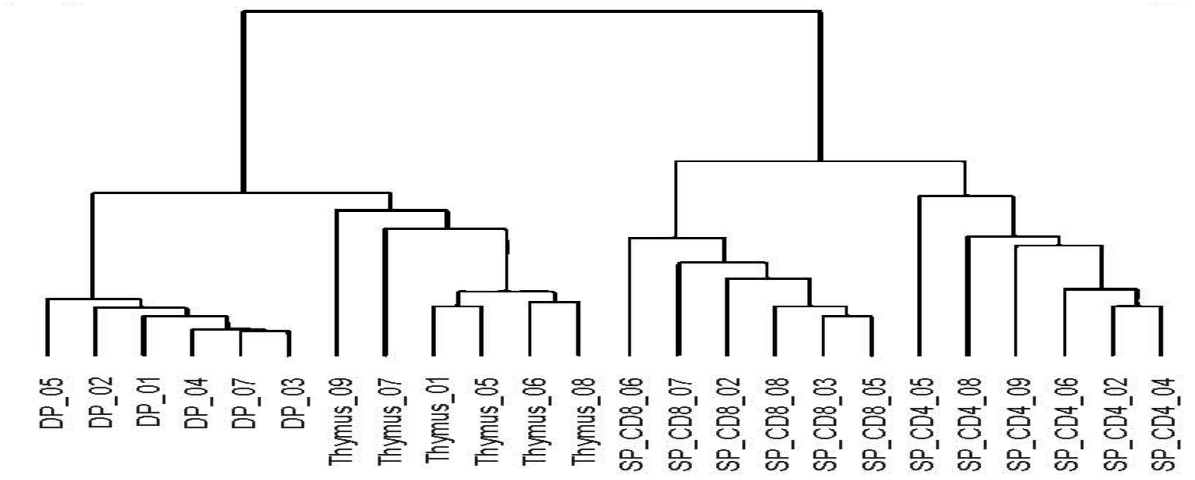

Figure 4.1 Hierarchial Clustering of different thymocyte sub populations. 6 samples for each thymic subpopulation have been used for analysis.

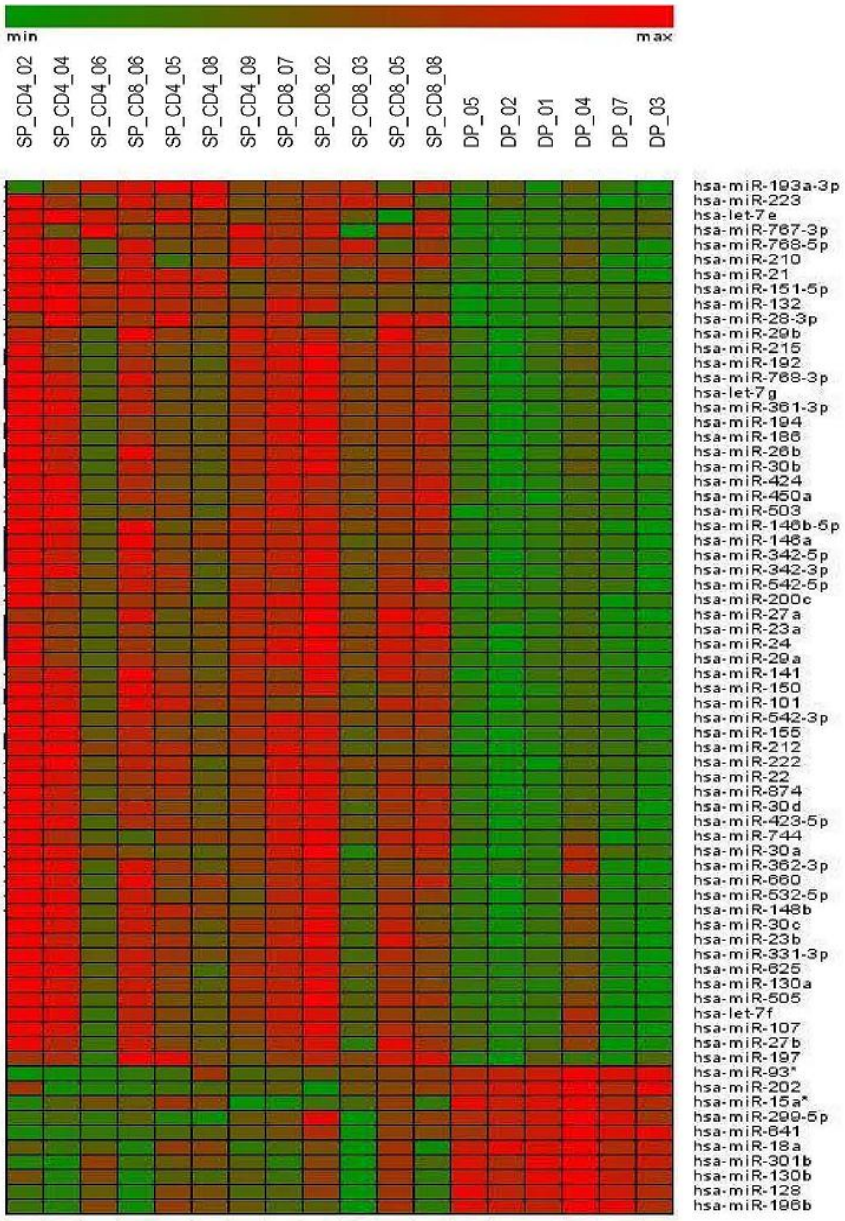

Figure 4.2 Differentially Expressed microRNAs between Double Positive and Single Positive thymocyte sub populations. Up regulated microRNAs are colored red and down regulated microRNAs are colored green. Notice that hsa-miR-150 is one of the most differentially regulated microRNAs.

4.2 Combined analysis of differentially expressed miRs and mRNAs in T cell development

miR-mRNA interactions may lead to degradation of the mRNA, integration of gene expression data with miR expression profiles can facilitate identification of genuine mRNA targets from the long list of potential targets provided by prediction algorithms. To exploit this approach, the mRNA expression profiles of T cells during their maturation from DP to SP $CD4^+$ cells were analysed using the HG-U133 Plus 2.0 platform (Affimetrix): 702 genes were identified as up-regulated in SP compared with DP, whereas 351 were down-regulated. The Functional Annotation Clustering tool implemented in the DAVID website <www.david.abcc.ncifcrf.gov> confirmed that a high number of these differentially expressed genes were involved in T-cell activation and differentiation (Table 4.1).

miRBase [75] and TargetScan [76] software packages were used to predict targets of miRs that were differentially expressed in SP $CD4^+$ versus DP cells. Comparison of the list of targets identified by both algorithms with the subset of genes observed to be differentially expressed during T-cell maturation yielded a group of candidate targets whose expression was inversely correlated to that of related miRs. Functional Annotation Clustering performed on this restricted subset of targets revealed an enrichment in genes involved in regulation of the cell cycle and mitosis (Table 4.2). Several of these targets were of potential interest because of their known role in the control of T-cell survival, proliferation, and differentiation as well as neoplastic transformation, including *NOTCH3*, *BCL2L1*, *CDKN2D*, and *NFKB1*. NOTCH3, in particular, was experimentally validated as target of hsa-miR-150 by Margherita Ghisi [77].

Functional Annotation Clustering of differentially expressed genes

Annotation term	*P*	False discovery rate
Annotation cluster 1 Enrichment score: 9.08		
Cell activation	3.3×10^{-13}	6.0×10^{-10}
Leukocyte activation	8.6×10^{-13}	1.5×10^{-9}
Lymphocyte activation	1.1×10^{-11}	2.0×10^{-8}
Lymphocyte differentiation	2.3×10^{-11}	4.1×10^{-8}
Leukocyte differentiation	3.8×10^{-11}	6.9×10^{-8}
Hematopoietic or lymphoid organ development	1.4×10^{-10}	2.6×10^{-7}
Immune system development	9.0×10^{-10}	1.6×10^{-6}
Hemopoiesis	1.6×9^{-13}	2.8×10^{-6}
T-cell activation	1.1×10^{-8}	2.0×10^{-5}
T-cell differentiation	1.1×10^{-8}	2.0×10^{-5}
B-cell activation	1.9×10^{-5}	3.4×10^{-2}
B-cell differentiation	9.9×10^{-5}	1.8×10^{-1}
Annotation cluster 2 Enrichment score: 8.09		
Immunoglobulin-like	2.5×10^{-15}	3.9×10^{-12}
Immunoglobulin-like fold	3.0×10^{-15}	4.8×10^{-12}
Immunoglobulin domain	5.6×10^{-11}	8.1×10^{-8}
Immunoglobulin V-set	2.1×10^{-4}	3.4×10^{-1}
Immunoglobulin subtype	3.0×10^{-4}	4.8×10^{-1}
Immunoglobulin	1.1×10^{-2}	1.3×10

Table 4.1 Functional Annotation Clustering of the genes differentially expressed between DP and SP stages of thymocyte differentiation.

Annotation term	p-value	False Discovery Rate
Annotation cluster 1 **Enrichment score: 4.72**		
Cell cycle	1.6e-7	2.7e-4
Mitotic cell cycle	2e-6	3.3e-3
Cell division	4.5e-6	5.8e-3
Cell cycle phase	8.5e-6	1.4e-2
Cell cycle processes	8.6e-6	1.4e-2
M phase	1.1e-5	1.8e-2
Nuclear division	2.3e-5	3.8e-2
Mitosis	2.3e-5	3.8e-2
M phase of mitotic cell cycle	2.7e-5	4.5e-2
Organelle fission	3.3e-5	5.5e-2
Regulation of mitotic cycle	2.3e-2	3.2e1
Annotation cluster 2 **Enrichment score: 3.29**		
Cell division	7.4e-5	1.2e-1
Cell cycle	3.1e-4	3.3e-1

Table 4.2 Functional Annotation Clustering of the candidate microRNA target genes differentially expressed between DP and SP stages of thymocyte differentiation.

PART 2 - Meta analysis of gene and microRNA expression data in T-ALL

4.3 Genes Differerentially Expressed between T-ALL and Normal Thymocytes

Differential expression analysis identified about 9000 probes differentially regulated between T-ALL and normal thymocytes corresponding to 6445 genes (at FDR < 0.01). Only genes with all the probes regulated in the same direction (up regulation or down regulation) were considered, reducing the number of genes to 6161 (3125 upregulated and 3036 down regulated).

4.4 TFs Differentially Expressed between T-ALL and Normal Thymocytes

Out of 6161 differentially regulated genes, 32 transcription factors, obtained from HTRIdb database, are up regulated above 2.5 fold in T-ALL when compared to normal thymocytes (Table 4.3A). Among these transcription factors TAL1 deserve special mention because it has been implicated in T cell acute lymphoblastic leukemia. GATA2 is also an extremely important transcription factor involved in thymocyte development. GATA2, CEBPA, TAL1 which are expressed in early stages of T cell development like ETP and DN1 needs to be switched off before beta selection occurs. ETS transcription factors are specifically up regulated between DN2 and DN3 stages indicating their precise role in thymocyte development. RUNX1 described as a key gene in T-ALL, is also involved in initiating T cell lineage development. Therefore, several transcription factors which are involved in thymocyte development may be involved in T cell pathogenesis.

Transcription Factors
NFIL3
NFE2
MAFF
ATF3
ERG
JUN
RORA
CEBPD
TAL1
ZEB2
E2F3
NFIC
GFI1B
GATA2
FOSL2
REST
CEBPB
RUNX1
NFAT5
TCF7L2
LRRFIP1
ETS2
DDIT3
RARA
ETS1
CEBPA
SPI1
MAX
PAX5
NFATC2
RXRA
STAT3

Table 4.3A. Transcription factors differentially regulated between T-ALL and normal thymocytes.

Tanscription Factors	Number of Transcription Factor Binding Sites
NFE2	444
ATF3	286
TAL1	741
GATA2	1104
ETS2	1236
ETS1	1982
STAT3	75
MAX	158
PAX5	481

Table 4.3B. Number of TFBS (Transcription factor binding sites) of 9 transcription factors among highly up and down regulated genes in the dataset. Transcription factors are selected from 32 transcription factors based on CONFAC output.

Notice in Table 4.3B that ETS1, ETS2, GATA2 has very high number of binding sites among the genes which indicate that they may play roles not only in T cell development but also in its neoplastic transformation.

4.5 Targets of MicroRNAs Differentially Expressed between T-ALL and Normal Thymocytes

By applying the procedure described in section 3.5, 7430 unique microRNA-gene target pairs (4185 genes) were obtained which also contained 143 experimentally validated microRNA-gene target pairs. Targets of hsa-miR-150 were also included as described in the methods section.

4.6 Integration of genes and microRNAs differentially expressed in T-ALL and Normal Thymocytes

4185 unique genes which were found to be targets of differentially regulated microRNAs were intersected with 6445 differentially regulated genes (>5 or < -5 fold) between T-ALL and Normal thymocytes. This intersection produced a list of 1561 genes.

Among these genes, both differentially regulated and targets of differentially regulated microRNAs, those with at least 10 binding sites for the 9 considered transcription factors (see Appendix 3) were selected. A list of 64 unique genes (216 unique gene-microRNA pairs) was obtained. Among 216 microRNA-gene pairs, 94 microRNA-gene pairs (61 unique genes) were present whose expression were anti-correlated (see Appendix 4).

A gene-microRNA network was created with these 94 microRNA-gene pairs, together with the transcription factors (Figure 4.3). In the constructed network, 67 out of 94 microRNA-gene pairs have down regulated genes in T-ALL compared to normal thymocytes, indicating that up regulated microRNAs may have significant role to play in T cell leukemogenesis. Down-regulation of these genes may also be mediated by transcription factors which may act co-operatively with the up regulated microRNAs to down regulate these genes. Some interesting loops, one with microRNA 22 and another with microRNA-206, were in the network. MicroRNA-22 targets both CEBPD and ETS2. ETS2 has binding sites in CEBPD. Similarly, microRNA-206 targets ETS1 and TAL1. TAL1 has binding sites for ETS2. These loops may have biological significance in the leukemogenesis process. Additionally, the CONFAC output (Appendix 3) revealed a group of genes which have all the 9 selected transcription factors binding sites on them (Table 4.4). In this group, there are few interesting genes (marked in bold in Table 4.4) which are also regulated by the microRNAs (see Table 4.5).

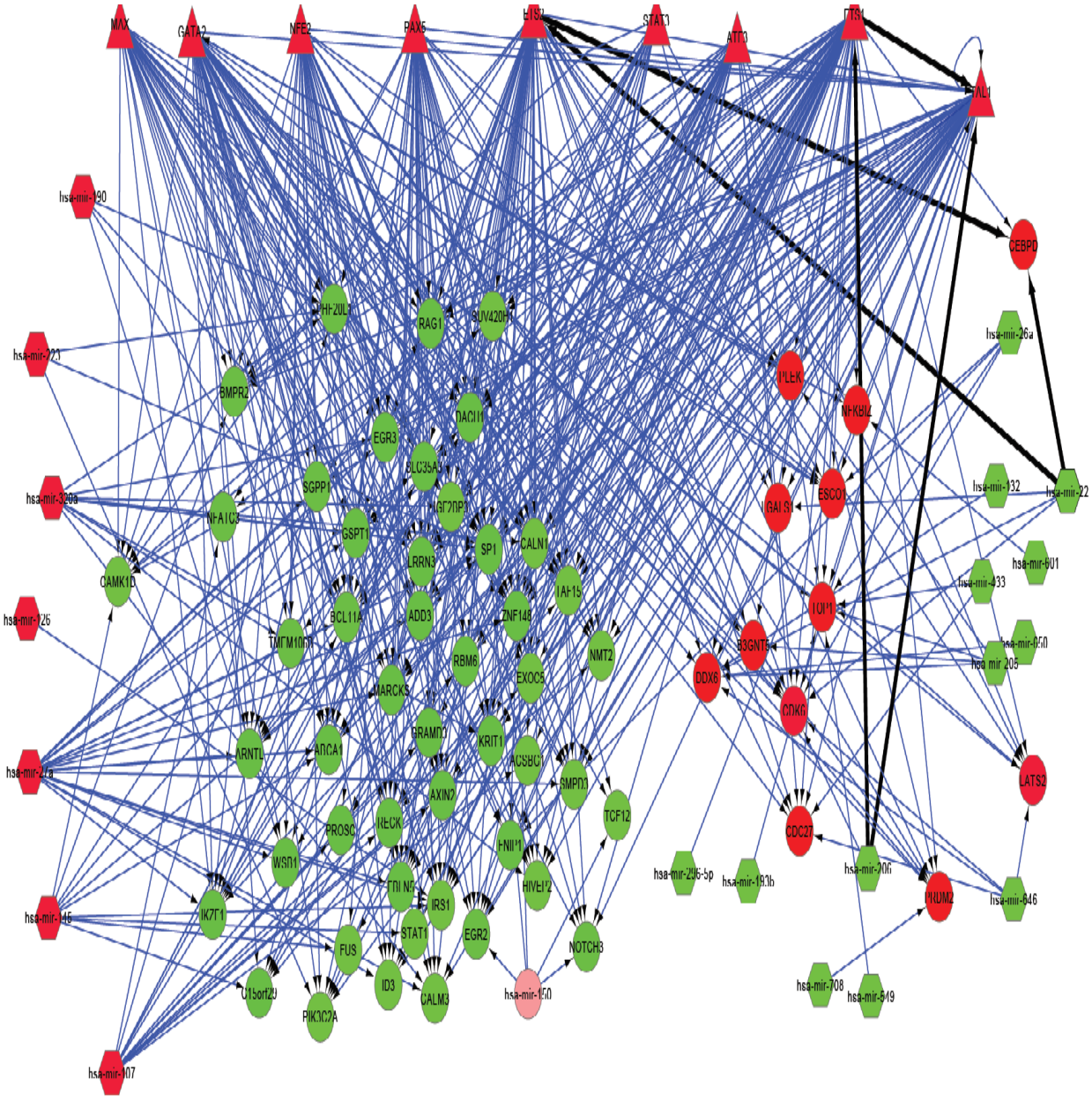

Figure 4.3. MicroRNA-Gene-Transcription factor Network in T-ALL. Triangles are transcription factors. Diamonds are microRNAs and round balls are genes. Red = Up regulated in T-ALL, Green = Down Regulated, Pink = hsa-mir-150 whose role is experimentally validated in Normal T cell Development. T-ALL compared to Normal thymocytes. In bold loop in the network.

Genes	NFE2	MAX	GATA2	STAT3	ATF3	TAL1	ETS2	ETS1	PAX5
BCL11A	13	5	18	3	6	15	28	61	20
BTBD5	5	5	23	1	5	16	23	43	11
NTNG2	4	3	22	3	3	13	22	35	7
LRRN3	13	1	19	2	7	8	23	33	2
ARNTL	4	1	26	1	4	16	19	28	4
IKZF1	9	2	11	1	2	10	24	36	6
IRS1	1	5	13	2	6	16	18	31	6
BMPR2	7	3	29	2	6	5	15	19	3
NFIL3	5	3	21	2	8	5	14	23	5
TNRC6B	9	4	5	1	4	8	16	26	6
ZNF148	3	1	14	1	1	8	13	24	7
ADD3	3	1	15	1	1	7	16	20	6
NIPBL	4	2	9	2	2	4	18	23	4
THRAP3	3	2	1	2	1	4	1	3	4
EDEM3	1	1	1	1	1	1	5	8	1

Table 4.4. Genes which have binding sites for all the 9 Transcription factors considered. Numbers indicate binding sites for the Transcription Factors.

Genes	MicroRNA	MicroRNA Sign	Gene Sign	Relationship
BCL11A	hsa-mir-107	POSITIVE	NEGATIVE	anti-correlated
LRRN3	hsa-mir-107	POSITIVE	NEGATIVE	anti-correlated
IRS1	hsa-mir-126	POSITIVE	NEGATIVE	anti-correlated
BMPR2	hsa-mir-145	POSITIVE	NEGATIVE	anti-correlated
BCL11A	hsa-mir-190	POSITIVE	NEGATIVE	anti-correlated
LRRN3	hsa-mir-190	POSITIVE	NEGATIVE	anti-correlated
BCL11A	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
IKZF1	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
IRS1	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
ZNF148	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
ADD3	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
ARNTL	hsa-mir-27a	POSITIVE	NEGATIVE	anti-correlated
IRS1	hsa-mir-145	POSITIVE	NEGATIVE	anti-correlated
ZNF148	hsa-mir-150	UNKNOWN	NEGATIVE	anti-correlated

Table 4.5. Targets of 9 Transcription factors regulated by microRNAs. Positive = Upregulation in T-ALL, Negative = Downregulation in T-ALL.

4.7 Additional Analysis on pathways

All the core enrichment genes (137 in number) related to different KEGG categories were collected (Appendix 2) and intersected with 4185 genes (7430 microRNA-gene pairs) to obtain 107 microRNA-gene pairs. Among these 107 microRNA gene pairs, 42 microRNA-gene pairs expression were found to be anti-correlated. (see Appendix 5). These 42 microRNA-gene pairs were used to construct a microRNA target enriched KEGG genes network.

Interestingly, two pathways, WNT Signaling Pathway and T cell Receptor Signaling pathway, were highly enriched with several upregulated microRNA targets. (Figure 4.6 and Table 4.7).

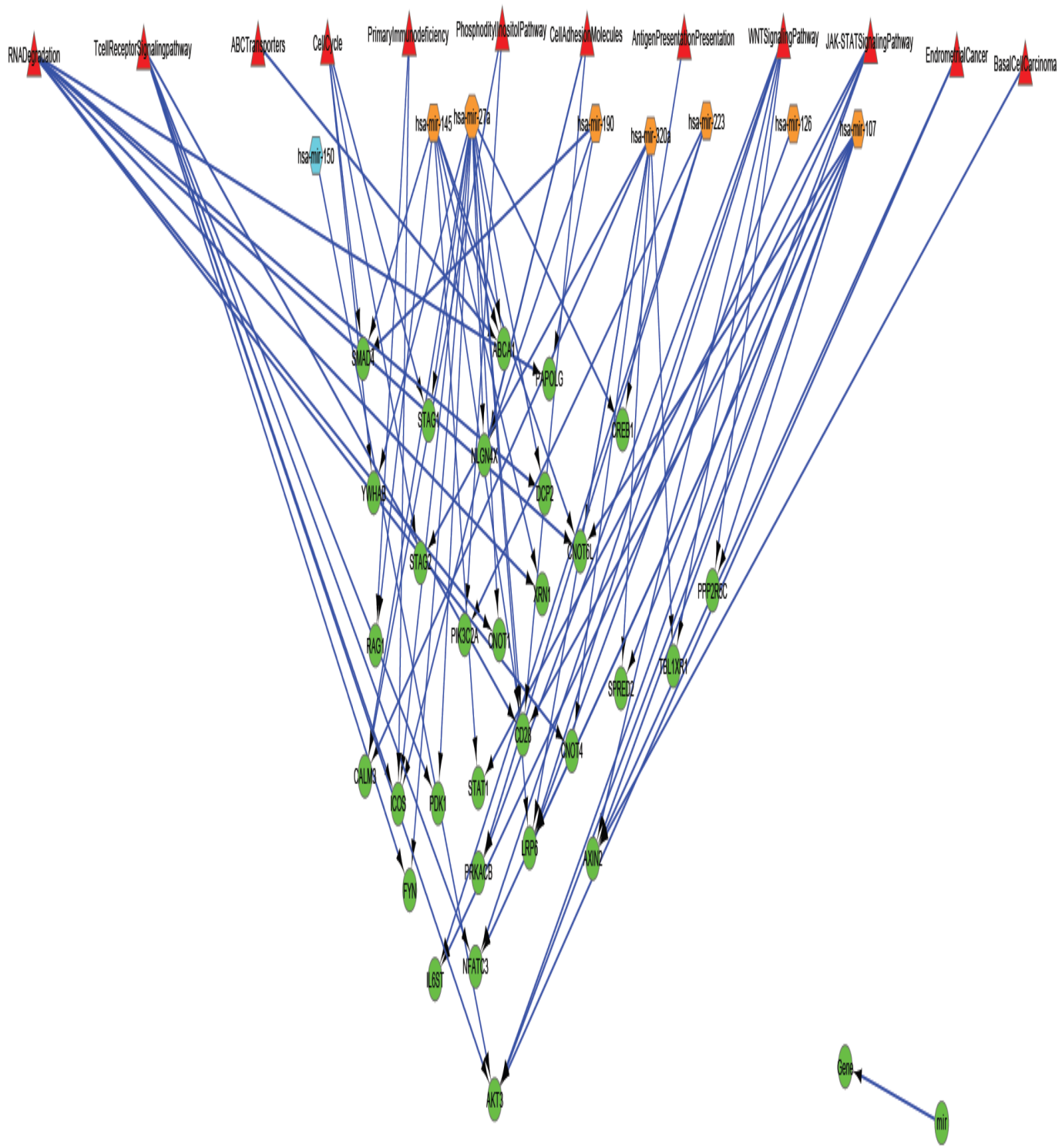

Figure 4.6. MicroRNA targeted GSEA core gene enriched network in T-ALL. In this network genes which are down regulated (colored green) are ball shaped, KEGG categories are red triangle, up regulated microRNAs in T-ALL are colored orange. Arrows indicate regulation.

GENES	MicroRNAs	Pathways
CNOT6L	hsa-mir-107	RNADegradation
PPP2R5C	hsa-mir-107	WNTSignalingPathway
AXIN2	hsa-mir-107	WNTSignalingPathway
LRP6	hsa-mir-107	WNTSignalingPathway
CD28	hsa-mir-107	TcellReceptorSignalingpathway
NFATC3	hsa-mir-107	TcellReceptor/WNTSignalingpathway
AKT3	hsa-mir-107	TcellReceptorSignalingpathway
LRP6	hsa-mir-126	WNTSignalingPathway
CNOT6L	hsa-mir-145	RNADegradation
CD28	hsa-mir-145	TcellReceptorSignalingpathway
SMAD4	hsa-mir-145	CellCycle
XRN1	hsa-mir-145	RNADegradation
SMAD4	hsa-mir-190	CellCycle
PAPOLG	hsa-mir-190	RNADegradation
PRKACB	hsa-mir-223	WNTSignalingPathway
IL6ST	hsa-mir-223	JAK-STATSignalingPathway
DCP2	hsa-mir-27a	RNADegradation
LRP6	hsa-mir-27a	WNTSignalingPathway
CNOT1	hsa-mir-27a	RNADegradation
ICOS	hsa-mir-27a	TcellReceptorSignalingpathway
PDK1	hsa-mir-27a	TcellReceptorSignalingpathway
CD28	hsa-mir-27a	TcellReceptorSignalingpathway
STAG1	hsa-mir-27a	CellCycle
YWHAB	hsa-mir-27a	CellCycle
FYN	hsa-mir-27a	TcellReceptorSignalingpathway
STAT1	hsa-mir-145	JAK-STATSignalingPathway
AKT3	hsa-mir-150	TcellReceptor/WNTSignalingpathway
CNOT6L	hsa-mir-320a	RNADegradation
TBL1XR1	hsa-mir-320a	WNTSignalingPathway
SPRED2	hsa-mir-320a	JAK-STATSignalingPathway
LRP6	hsa-mir-320a	WNTSignalingPathway
CNOT4	hsa-mir-320a	RNADegradation
STAG2	hsa-mir-320a	CellCycle

Table 4.7. Selected Pathways/categories which have at least 3 microRNAs targeting the genes of the pathways/ category. Down regulated genes are colored green, up regulated microRNAs are colored red. Each pathway is colored with different colors.

5. DISCUSSION

In the first part of the work, miR expression in human T-cell populations from sequential stages of maturation was profiled using microarrays. All the results presented were published in [77]. This is the first comprehensive analysis of miR regulation performed in human thymocytes as all previous studies were conducted either in mouse models or in peripheral T lymphocytes at later stages of development. Results indicate that each human thymocyte subpopulation (DP, SP $CD4^+$, SP $CD8^+$) is characterized by a distinct miR expression profile, with the pattern of miR expression reflecting the developmental relationship between maturation stages (Figure 4.1). A general trend was detected in up-regulated miR expression in the maturation from the DP to SP stage of thymocyte development. This result is consistent with previous studies that correlated miR levels to cell differentiation [55] and suggests that miR up-regulation in this context could be important for down-regulating genes associated with an immature phenotype. Moreover, because immature thymocytes at the DP stage are subjected to both positive and negative selection through T-cell receptor-activated apoptotic/survival signals, the up-regulation of miRs during the maturation to SP cells could also play a role in suppressing genes involved in this selection process [78].

Interestingly, some of the microRNAs which have been reported to play an important role in the differentiation of the lymphoid (miR-150, miR-155, miR-23a) or myeloid (miR-146, miR-223) hematopoietic lineages [42, 79, 80] and in the regulation of cellular proliferation or apoptosis (miR-150, miR-23a, miR-27a, miR-24) were up regulated in single positive stage of thymocyte development [81, 82]. Among these microRNAs, hsa-miR-150 was experimentally validated as regulator of NOTCH3, a down regulated gene in SP compared to DP.

In the second part of the work, gene expression profile of normal thymocytes was compared with that of T cell acute lymphoblastic leukemia. Differentially regulated microRNAs between normal thymocytes and T cell acute lymphoblastic samples were collected from scientific literature and combined with gene expression data to construct a network.

For many years the main interest of the researchers was focused on discovering gene signatures specific to a particular T-ALL subtype based on a specific fusion gene product [83-85]. Indeed, different subtypes of T-ALL are associated with chromosomal translocations between gene codifying T cell receptors and oncogenic transcription factors forming fusion gene products. The meta-analysis showed several up regulated transcription factors in T-ALL compared to normal thymocytes independently of T-ALL subtype (Table 4.3A). Most of the up regulated transcription factors are not fusion gene products in T-ALL but are involved in early stages of T cell development in the thymus. Interestingly, a recent paper by Saida Dadi et.al [86] reports the interaction of TLX1 (fusion product oncogenic transcription factor in T-ALL) and ETS1 (Transcription Factor involved in early stages of T cell development in thymus) as a mechanism causing T cell maturation arrest and leukemogenesis in T-ALL. The results showed in Table 4.3A clearly present a group of transcription factors which may play role in leukemogenesis irrespectively of the T-ALL subtype.

A TF-gene-microRNA network was then constructed, involving 9 TFs that were targeting strongly differentially expressed genes (Figure 4.3). In the network , there are seven up regulated microRNAs,

which are found to target down regulated genes, and among them microRNA 223 was known to be upregulated in T-ALL [60, 61]. MicroRNA-27 is implicated in myeloid cell differentiation l [87] and is highly upregulated in SP thymocytes (Figure 4.2).

Among the down regulated microRNAs in the network, hsa-miR-22, hsa-miR-26a, hsa-miR-132 may be interesting candidate microRNAs for further functional studies on T-ALL as they are also down regulated in immature normal T cell phenotype (DP) compared to SP thymocyte populations.

T cell Acute lymphoblastic leukemia arise mainly from normal T cells corresponding to either double negative (LYL1+), early cortical (HOX11) or late cortical (TAL1) stages of T cell development [83]. This suggests that T-ALL can develop from immature T cell phenotype. Interstingly, ETS2, a transcription factor which we found to be up regulated in the T-ALL, was also up regulated in very early stages of thymocyte development. Moreover, a microRNA down regulated both in T- ALL and DP thymocytes, hsa-miR-22 was predicted to regulate this transcription factor. It is known that hsa-miR-22 is a tumour suppresor and is down regulated in different types of cancer [88, 89, 90]. It thus may be interesting to clarify the functional role of hsa-miR-22 in T-ALL.

Further improvement of the meta analysis will be performed and will become the starting point of future biological validation.

APPENDIX 1

Selection of interesting transcription factors and their target genes from literature

Several authors for the last 10 years have extensively used high throughput genomic technologies to understand the oncogenic transcription factor direct gene targets or specific genes related to different transcription factors in T- ALL. In this work, "transcription factor associated genes " were collected from the literature (7 published articles) in the following manner.

1) 27 human T-ALL samples expressing either high levels of HOX11 or TAL1 or LYL1 were used for microarray analysis by Ferrando et.al [83]. In the main article, they have highlighted several "top genes among 200 nearest neighbours for each transcription factor oncogenes". These transcription factor specific genes were collected for the meta analysis.

2) PER-117 T-ALL cell line was used by Katrin Hoffmann et.al [91] to stably transfect HOX11 transcription factor. Subsequently microarray analysis was performed to identify differentially regulated genes as a result of HOX11 over expression. All the genes reported to be differentially regulated in the main paper were assembled for meta analysis.

3) Teresa Palomero et.al [92] identified 71 genes whose promoters may be occupied by oncogenic transcription factor TAL1 by ChIP on chip experiments in Jurkat cells. They further categorised these 71 genes into different functional categories. All the genes were collected except those belonging to " Unknown Class" for the purpose of robust meta analysis.

4) In the year 2007, Emmanuelle Clappier et.al [85]reported a gene cluster specific to TCRB-MYB translocation in very young children. In the microarray analysis this cluster was identified

specific for 4 TCRB-MYB translocation patients. This reported gene cluster was also included in the analysis.

5) Kim de Keersmaecker et.al in the year 2010 [84], performed an interesting analysis with T cell acute lymphoblastic leukemia human samples. They at first discovered a mouse T-ALL gene signature then performed a GSEA analysis with this signature on human T-ALL samples. All the "leading edge genes" were collected from the supplementary data of the paper.

6) Adam. A. Margolin et.al [93] in the year 2009 reported in their paper a list of validated genes (by ChIP or qPCR) which are targets of NOTCH1, HES and MYC transcription factors. The gene set reported by them is included in the meta analysis list.

7) Finally a list of "high confidence" targets of TAL1 (some of them are also targets of cofactors HEB, RUNX1,GATA3 and E2A transcription factors) are collected from the recently published paper of Takaomi Sanda [94] and included in the list for the purpose of meta analysis.

After the collection of data, it was necessary to look for 1) If any gene is reported in multiple papers or 2) If a gene is regulated by multiple transcription factors or is specific to upregulation of multiple transcription factors. For this purpose a matrix is constructed. If a gene is reported in a publication gets a score 1. Addition of the scores for each gene across 7 publications, gives the total score of a gene. The genes with high scores (> 1) are important as they are occurring multiple times in the collected data and may have very significant role to play in T cell oncogenesis.

Out of 635 genes collected from the seven chosen papers, 19 genes were found to be reported in more than one paper (see Table A1.1). RUNX1 tops the list - mentioned in three key papers. Remaining genes are reported in two articles. RUNX1 was described to be a TAL1 specific gene [83]. It was also one of the genes which was present in the human TLX transcription factor signature [84].

Finally RUNX1 promoter was also reported to be bound by TAL1, HEB, E2A, GATA3 transcription factors [94]. Another gene which was found to be regulated by all these transcription factors was SELL which was also described to be a LYL1 specific gene [83]. CCNA2 and TOP2A are HOX11 transcription factor (TLX1) [83] specific genes and also found to be up regulated in patients having TCRB-MYB translocation. ITGAL is a gene found to be specific for both TLX transcription factor [84] as well as TAL1 transcription factor [83].

BCL2, an anti apoptotic gene in lymphocytes, was shown to be specific for LYL1 transcription factor and was also found to be up regulated with the enforced over expression of HOX11(TLX1) [91]. Histone cluster HIST1H2K is also another gene which is highly expressed with the overexpression of HOX11 and was found to be an experimentally validated target of MYC [93].NR4A3 is another gene upregulated with the overexpression of HOX11 and is also a direct target of TAL1 (ChIP on chip) [25]. TCF7 is specific for TLX1 and regulated by TAL1 where as KIF11 is up regulated in TCRB-MYB translocation patients and is a human TLX transcription factor specific gene. TOX, ADAM10, TSPAN2, STAT5A,STAT5B, RASGRP1,KIF5B, REEP5 are the genes bounded by TAL1 and its cofactors and are also human TLX transcription factors specific.

Therefore in conclusion there are a set of genes in T-ALL patients which are under the control of several transcription factors (whose roles are well studied in T-ALL). Expression levels of these genes are probably dependent on the combined effect of activating or repressing

function of these transcription factors. These genes are important to be considered as potential targets of therapy as they are target genes of more than one transcription factor implicated in T- ALL.

Gene	Adolfo Ferrando (2002)	Katrin Hoffmann (2004)	Teresa Palomero (2006)	Emmanuelle Clappier (2007)	Kim De Keersmaecker (2010)	Adam Margolin (2009)	Takaomi Sanda (2012)	Score
RUNX1	1	0	0	0	1	0	1	3
CCNA2	1	0	0	1	0	0	0	2
TOP2A	1	0	0	1	0	0	0	2
TAL1	1	0	0	0	0	0	1	2
ITGAL	1	0	0	0	1	0	0	2
SELL	1	0	0	0	0	0	1	2
BCL2	1	1	0	0	0	0	0	2
HIST1H2K	0	1	0	0	0	1	0	2
NR4A3	0	1	1	0	0	0	0	2
TCF7	0	0	1	0	1	0	0	2
KIF11	0	0	0	1	1	0	0	2
TOX	0	0	0	0	1	0	1	2
ADAM10	0	0	0	0	1	0	1	2
TSPAN2	0	0	0	0	1	0	1	2
KIF5B	0	0	0	0	1	1	0	2
STAT5B	0	0	0	0	1	0	1	2
RASGRP1	0	0	0	0	1	0	1	2
REEP5	0	0	0	0	1	0	1	2
STAT5A	0	0	0	0	1	0	1	2

Table A1.1. Genes which are reported to be specific for more than one transcription factor among 7 collected T-ALL related articles.

APPENDIX 2

Gene Set Enrichment Analysis (GSEA)

GSEA software (in the GseaPreranked version) was used together with Kegg database (ftp.broadinstitute.org://pub/gsea/gene_sets/c2.cp.kegg.v3.1.symbols.gmt). Ranked file used in the analysis was the list of differentially regulated genes arranged in the descending order of fold change. Gene set enrichment analysis is based on an "enrichment score" which indicates to what degree a particular pathway (gene set) is over representated at the top or the bottom of the ranked list. Enriched Pathways which have "q- FDR value < 0.1" are reported.

Gene Set Enrichment Analysis identified 14 gene set categories (which have FDR value less than 0.1) likely to be involved in T cell acute lymphoblastic leukemia. These are KEGG_ Haematopoetic Cell lineage (35 genes), KEGG_ T cell Receptor Signaling pathway (55 genes), KEGG _Primary Immunodeficiency (15 genes), KEGG_Cell Adhesion Molecules (46 genes), KEGG_ Antigen Processing and Antigen (23 genes), KEGG_ ABC Transporters (16 genes), KEGG_RNA Degradation (29 genes), KEGG_ Arachidonic Acid Metabolism (18 genes), KEGG_JAK STAT signaling pathway (51 genes), KEGG_ Endrometrial Cancer (23 genes), KEGG_Phosphatidylinositol Signaling (34 genes), KEGG_Basal Cell Carcinoma (23 genes), KEGG_Cell Cycle (50 genes), KEGG_WNT Signaling pathway (66 genes). Enrichment plots for the KEGG gene sets are composed of core enrichment genes which are mainly present at the bottom of the ranking list (negative -correlation) indicating a trend in down regulation of several key genes in T-ALL as compared to normal thymocytes.

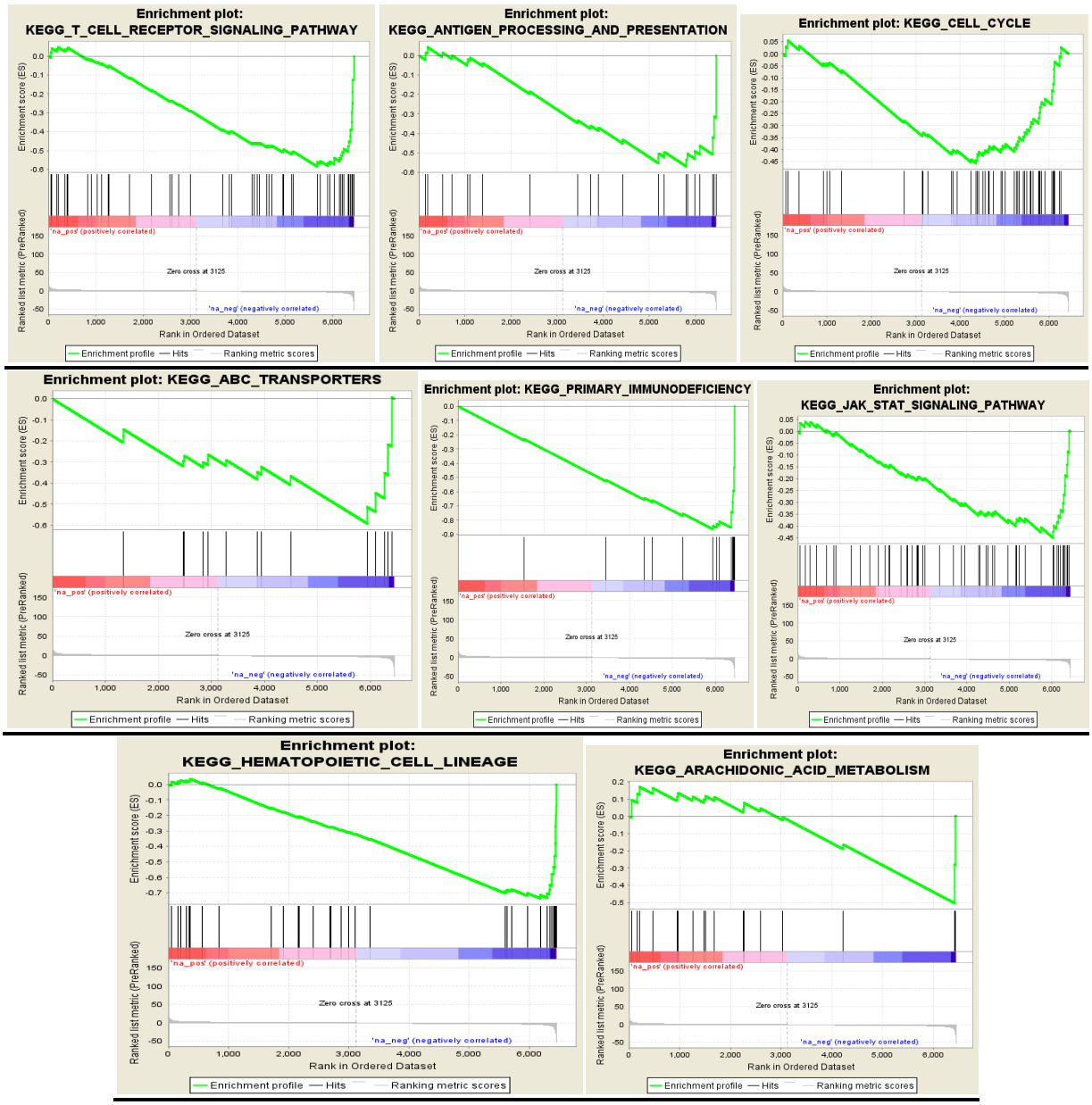

Figure A2.1 Gene Set Enrichment plots for the 14 identified gene set categories.

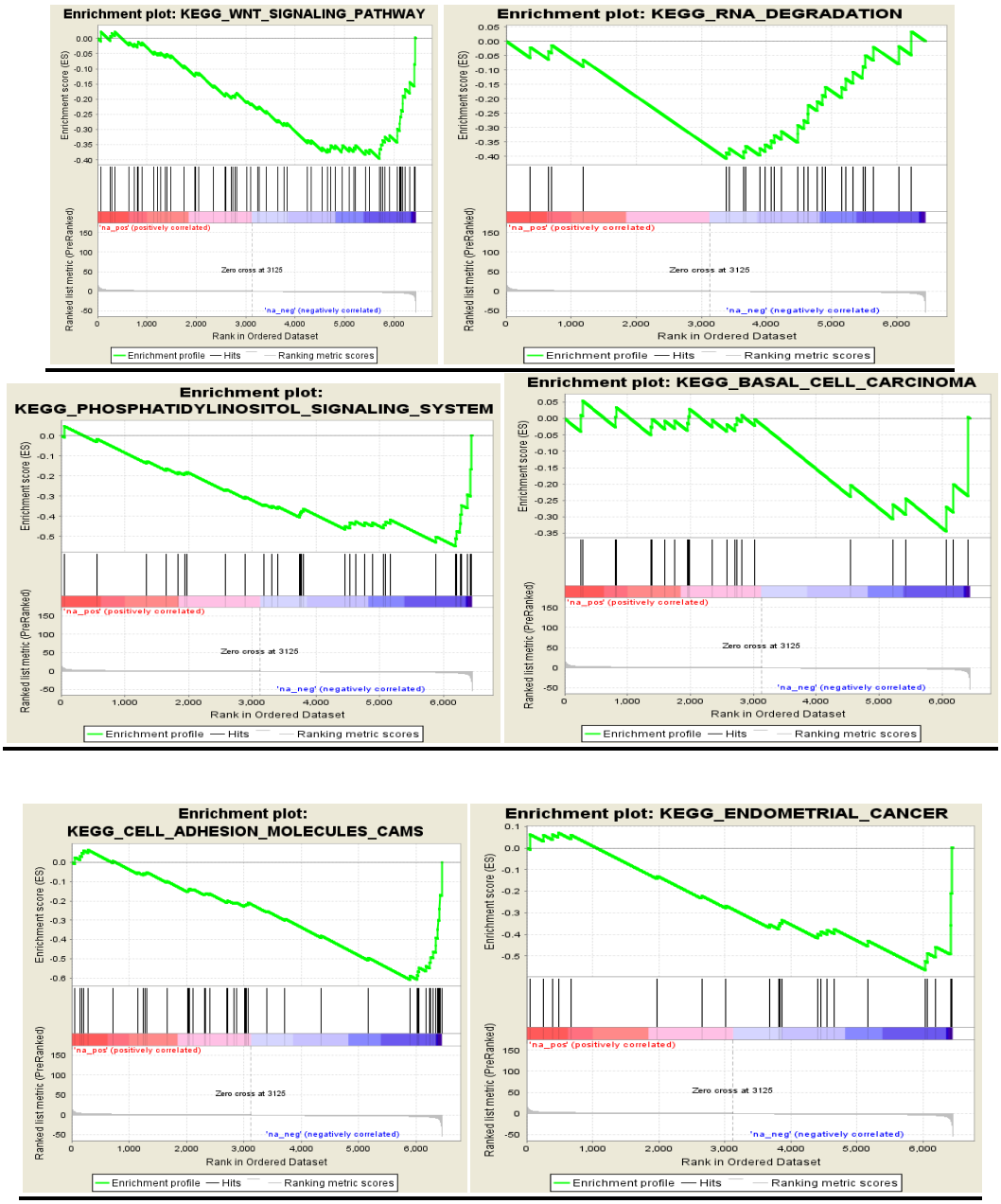

Figure A2.1 Gene Set Enrichment plots for the 14 identified gene set categories.

APPENDIX 3

Confac software was used to obtain the following table. For each differentially expressed gene is reported the number of predicted transcription factor binding sites (for each of the 9 considered transcription factors).

Genes	NFE2	MAX	GATA2	STAT3	ATF3	TAL1	ETS2	ETS1	PAX5	sum
BCL11A	13	5	18	3	6	15	28	61	20	169
SMPD3	9	1	20	0	7	17	28	57	18	157
DACH1	9	2	39	0	6	14	25	41	7	143
BTBD5	5	5	23	1	5	16	23	43	11	132
HIVEP2	6	2	26	0	8	13	23	44	8	130
MTSS1	5	0	16	2	8	14	28	45	7	125
NTNG2	4	3	22	3	3	13	22	35	7	112
LRRN3	13	1	19	2	7	8	23	33	2	108
DAAM1	8	2	27	0	2	17	13	28	8	105
ARNTL	4	1	26	1	4	16	19	28	4	103
ELK3	8	0	12	3	6	8	22	37	6	102
IKZF1	9	2	11	1	2	10	24	36	6	101
IRS1	1	5	13	2	6	16	18	31	6	98
SKAP1	5	0	21	0	4	4	26	33	3	96
EGR3	5	6	5	0	2	13	13	31	15	90
BMPR2	7	3	29	2	6	5	15	19	3	89
NFIL3	5	3	21	2	8	5	14	23	5	86
DFNB31	6	1	11	0	3	12	18	29	5	85
TSPAN7	7	0	19	0	5	6	17	22	8	84
PTEN	8	6	18	0	5	10	12	17	3	79
TNRC6B	9	4	5	1	4	8	16	26	6	79
ZNF148	3	1	14	1	1	8	13	24	7	72
MARCKS	3	4	12	0	2	14	10	16	9	70
ADD3	3	1	15	1	1	7	16	20	6	70
FCER1G	6	0	5	1	1	11	16	21	8	69
NIPBL	4	2	9	2	2	4	18	23	4	68
EIF4A1	4	2	3	0	4	9	11	21	10	64
CAMK1D	3	4	9	0	2	9	11	20	6	64
CD247	6	0	13	0	1	8	12	19	4	63
TCF8	4	0	10	1	5	6	14	17	3	60

HS3ST3B1	2	2	11	0	1	8	11	21	2	58
ACPL2	6	1	11	0	3	6	7	16	5	55
STRN	4	0	12	0	3	3	12	19	0	53
IGFBP7	3	0	12	2	4	4	10	12	6	53
DGKA	4	0	3	3	1	7	13	16	3	50
RAG1	1	0	15	0	2	6	7	14	4	49
SCRN1	4	2	12	0	1	3	10	14	2	48
HIPK1	2	2	14	0	3	6	9	11	1	48
ABCA1	5	3	4	0	1	9	7	14	3	46
AXIN2	1	0	10	0	0	10	7	12	6	46
IGF2BP3	0	6	3	0	0	7	9	17	3	45
CALM3	5	0	10	0	0	8	3	11	8	45
IL23A	0	0	3	0	5	9	11	13	3	44
SP1	2	1	3	0	1	3	10	19	5	44
HHEX	3	0	5	0	1	4	13	15	2	43
RASSF3	3	0	8	1	1	6	11	12	1	43
MAN2A1	4	0	14	1	2	2	8	11	1	43
ZNF638	3	0	8	0	4	2	7	16	1	41
IRX5	3	1	2	0	2	7	6	15	5	41
KIAA1212	1	1	4	0	3	5	12	13	2	41
SRPK2	3	1	16	0	3	2	6	8	0	39
ID3	2	0	3	0	4	4	10	14	2	39
WASL	3	0	8	1	4	7	4	7	5	39
CKAP4	3	0	1	0	0	5	12	15	3	39
ABCD3	5	0	12	0	0	1	8	12	1	39
ITGAV	5	0	11	0	0	1	9	11	2	39
RECK	1	1	11	0	1	4	8	11	1	38
NFE2	0	0	8	0	3	3	6	16	2	38
CDK6	2	2	3	0	3	4	8	11	4	37
CALN1	3	1	11	0	0	5	6	7	4	37
NPEPPS	2	0	11	0	3	7	3	7	4	37
BCL2L1	2	2	1	0	2	4	7	11	7	36
TUBD1	0	0	2	0	0	6	7	14	6	35
BTBD11	3	0	6	0	0	5	6	11	3	34
WNK1	4	0	0	0	0	7	7	13	3	34
MAFF	2	3	0	0	2	5	9	11	1	33
PTPRM	6	0	7	1	2	4	4	7	1	32
LYN	3	0	6	1	2	2	8	9	1	32
TAL1	1	0	6	0	0	8	4	9	4	32
S100A4	1	0	0	3	0	7	5	10	6	32
GSPT1	0	2	4	2	0	5	5	8	4	30

ACTR2	3	0	8	0	1	4	5	7	1	29
BHLHB2	3	4	0	0	3	1	3	7	7	28
ITPR2	0	0	11	0	2	0	5	9	1	28
NMT2	4	0	13	0	0	3	2	3	2	27
DDX6	0	0	8	0	0	2	5	12	0	27
FLJ22531	2	0	3	0	0	4	6	11	1	27
C15orf29	0	0	7	1	1	2	6	9	0	26
RAG2	5	0	6	0	2	1	5	7	0	26
EWSR1	3	2	2	0	2	4	4	7	2	26
RUFY3	1	0	6	0	0	3	7	8	1	26
ATF7IP	3	2	6	0	0	3	4	6	1	25
FNIP1	0	2	6	0	0	2	6	9	0	25
JUN	0	0	2	0	2	4	4	7	5	24
ADAM10	2	0	5	0	3	3	5	6	0	24
SLAMF1	3	0	2	2	0	1	6	8	1	23
ALAS2	2	1	6	2	0	1	4	6	1	23
JAK1	1	0	5	0	3	4	3	7	0	23
AQP3	2	0	3	0	0	2	7	8	1	23
PCAF	1	1	0	1	0	3	7	10	0	23
KRIT1	1	0	0	1	0	3	5	9	4	23
TAF15	2	1	2	0	4	3	2	5	3	22
FBLN5	3	0	1	1	2	2	6	6	1	22
ASXL2	1	2	4	0	1	5	1	3	4	21
CBFA2T2	1	0	3	0	2	3	3	6	3	21
TPM4	1	0	1	0	4	3	4	8	0	21
THRAP3	3	2	1	2	1	4	1	3	4	21
PLXDC1	3	2	1	0	2	2	3	6	1	20
CTHRC1	2	3	0	0	0	3	3	6	3	20
RASSF6	2	0	2	2	0	1	6	6	1	20
SLC35A3	0	0	7	0	0	2	4	7	0	20
S100A9	1	0	1	0	0	2	4	10	2	20
EDEM3	1	1	1	1	1	1	5	8	1	20
COG3	2	2	4	0	1	5	3	3	0	20
SPEN	2	0	5	1	0	2	2	8	0	20
MTA3	1	0	3	0	1	3	4	6	1	19
EXOC5	1	0	0	1	0	2	5	8	2	19
LRRC16	3	0	4	0	0	4	1	4	3	19
ELOVL4	1	1	1	0	4	1	3	4	3	18
STK38	1	0	3	0	0	3	4	6	1	18
CD4	3	0	4	0	0	1	5	5	0	18
PRDM2	4	1	2	0	2	0	4	4	1	18

CDC27	0	0	8	0	3	4	1	1	1	18
SLC4A1	2	0	5	0	0	2	2	3	4	18
EGR2	2	0	2	1	2	1	4	5	1	18
TCF7	0	0	4	0	0	2	3	5	3	17
SOS1	1	1	3	0	1	0	3	7	1	17
PAFAH2	0	0	7	0	0	2	3	4	1	17
IKZF3	2	0	7	0	1	0	3	4	0	17
ITPKB	2	0	3	0	0	3	2	6	1	17
ALOX5	0	0	2	0	1	1	6	7	0	17
CTLA4	1	0	3	0	0	1	5	6	0	16
PLEK	1	0	5	0	0	1	4	4	1	16
RASGRP1	0	0	7	0	0	3	2	3	1	16
G0S2	3	2	3	0	2	0	2	2	1	15
MGC29671	2	0	0	0	1	1	4	5	2	15
RBM6	0	0	0	0	1	3	3	3	5	15
MMP9	1	0	1	0	0	1	5	6	1	15
HIST1H1E	0	0	5	0	2	0	1	4	2	14
CMTM7	1	0	1	0	0	2	4	4	2	14
DOK3	5	0	0	0	1	1	2	3	2	14
NAPE-PLD	1	0	5	0	0	3	2	3	0	14
CD6	2	0	0	0	1	1	4	5	1	14
NOTCH3	1	2	0	0	0	3	2	5	1	14
EDEM1	0	0	1	1	1	2	4	5	0	14
PTPRC	2	0	2	1	1	0	4	4	0	14
YME1L1	1	0	2	0	1	0	3	6	0	13
DSG2	0	0	4	0	0	0	4	5	0	13
ESCO1	1	1	1	0	0	2	3	4	1	13
MAN1C1	2	0	1	0	0	5	0	2	3	13
TSHR	3	0	4	1	0	1	1	1	2	13
PHF3	1	0	5	0	2	0	0	4	1	13
ITK	0	0	4	0	2	0	2	3	2	13
RIPK4	0	0	2	0	1	3	2	2	3	13
LATS2	0	2	1	1	2	0	3	3	0	12
FLJ20125	0	0	2	2	0	0	3	5	0	12
INSIG2	1	0	0	1	0	1	3	5	1	12
PDE7A	2	0	5	0	0	1	2	2	0	12
ITGAM	0	0	2	0	0	0	4	6	0	12
PIK3C2A	1	0	1	1	2	1	2	3	0	11
BCL2L11	0	1	0	0	2	3	1	2	2	11
CLEC11A	0	0	0	1	0	0	2	6	2	11
ANXA1	1	0	1	0	0	0	4	5	0	11

CFLAR	2	1	0	0	3	1	2	2	0	11
BCL2A1	1	0	2	1	0	1	2	4	0	11
MUC20	0	0	1	0	0	2	3	4	1	11
IL21R	1	0	0	0	2	2	3	3	0	11
SLC29A1	0	2	2	0	0	2	2	2	0	10
GGA2	1	1	0	0	1	4	1	2	0	10
VPREB1	0	0	1	0	0	2	2	4	1	10
TYROBP	0	0	0	0	0	1	3	6	0	10
SATB1	2	0	2	0	0	0	2	3	1	10
KIAA0980	3	0	1	0	1	0	2	3	0	10
IL1A	1	0	2	0	0	1	2	3	0	9
MYO7B	0	1	2	0	0	0	3	3	0	9
ITGA4	0	5	2	0	0	0	1	1	0	9
AGXT2L2	1	0	1	0	0	1	0	2	3	8
ANXA3	0	0	3	0	0	0	2	3	0	8
STK32B	0	0	0	0	0	2	3	3	0	8
FTH1	0	0	4	0	0	1	1	1	1	8
BLVRB	0	0	1	0	1	0	0	2	4	8
STOM	1	0	1	0	0	2	1	2	1	8
TPP2	0	2	0	0	0	1	2	3	0	8
NFKBIZ	0	0	2	0	0	0	2	3	0	7
COCH	1	0	1	0	0	0	1	2	2	7
LGALS1	0	0	0	1	0	4	1	1	0	7
TIMP1	2	0	0	0	0	2	1	1	0	6
SLC7A6	0	1	0	0	1	2	1	1	0	6
ID1	2	0	0	0	3	1	0	0	0	6
SUV420H1	0	0	0	0	0	3	1	2	0	6
CSNK1A1	2	0	0	0	0	1	1	1	1	6
TOP1	0	3	0	0	1	1	0	1	0	6
LTF	1	0	0	0	0	2	1	2	0	6
WSB1	1	1	0	0	0	0	1	3	0	6
SGPP1	1	0	3	1	0	1	0	0	0	6
PHF20L1	0	3	2	0	0	0	0	0	1	6
BPI	0	0	0	0	0	3	1	2	0	6
ACSBG1	0	0	0	0	0	0	2	3	0	5
CCL5	1	1	0	0	0	0	1	2	0	5
IL7R	1	0	0	1	0	0	1	2	0	5
STX17	0	0	3	0	1	0	0	1	0	5
TCF12	0	0	3	0	0	0	0	0	2	5
SENP7	0	0	0	0	0	0	1	2	2	5
B3GNT5	1	0	2	0	0	0	1	1	0	5

TMEM63A	0	0	1	0	0	1	1	1	1	5
MPO	0	0	1	0	0	0	2	2	0	5
SLA	0	0	2	0	0	1	1	1	0	5
ELA2	1	0	1	0	1	1	0	0	0	4
DNMBP	1	0	0	0	0	1	1	1	0	4
MYO1F	0	0	0	0	0	0	2	2	0	4
CHRNA3	0	0	0	0	0	1	1	1	1	4
CDA	1	0	0	0	0	0	1	2	0	4
NFATC3	0	0	0	0	0	0	2	2	0	4
CIRBP	2	0	0	1	0	1	0	0	0	4
CST7	1	0	1	0	0	0	1	1	0	4
LARS	0	0	1	0	0	0	1	1	1	4
ATM	0	0	2	0	1	0	0	0	0	3
TMEM106B	1	0	1	0	0	0	0	1	0	3
HERC4	0	1	1	0	0	0	0	1	0	3
HBB	0	0	1	0	1	0	0	1	0	3
LCN2	0	0	0	0	0	1	1	1	0	3
NKG7	0	0	0	0	0	1	1	1	0	3
GRAMD3	0	0	0	0	0	0	1	1	1	3
HERC4	0	1	1	0	0	0	0	1	0	3
MMP8	1	0	0	0	0	0	1	1	0	3
STAT1	0	0	0	0	0	0	1	2	0	3
PYGL	0	0	1	0	0	0	1	1	0	3
AP3D1	1	2	0	0	0	0	0	0	0	3
MS4A3	0	0	0	0	0	0	1	1	1	3
PROSC	0	0	0	0	1	0	0	1	1	3
TMEM33	1	0	1	0	1	0	0	0	0	3
TIMP2	0	0	0	0	0	0	1	1	0	2
ARG1	0	0	2	0	0	0	0	0	0	2
MTMR1	0	0	1	0	0	1	0	0	0	2
SERPINA1	0	0	0	0	0	0	1	1	0	2
CEBPD	0	0	0	0	0	0	1	1	0	2
FUS	0	0	0	0	0	0	0	1	1	2
CHI3L1	1	0	0	0	0	0	0	1	0	2
S100A8	0	0	0	0	0	2	0	0	0	2
SF1	0	0	0	0	0	1	0	0	1	2
MDM4	0	0	0	0	0	0	0	2	0	2
KIAA0100	0	0	2	0	0	0	0	0	0	2
DPP4	1	0	0	0	0	0	0	0	0	1
LY9	0	0	0	0	0	0	0	1	0	1
ASPM	0	0	0	0	0	1	0	0	0	1

TOP2A	1	0	0	0	0	0	0	0	0	1
TRIM58	0	0	0	0	0	0	0	1	0	1
CD8A	0	0	1	0	0	0	0	0	0	1
C2orf43	0	0	1	0	0	0	0	0	0	1
EPHX2	0	0	0	0	0	1	0	0	0	1
CD8B	0	0	0	1	0	0	0	0	0	1
TMEM168	0	0	0	0	0	1	0	0	0	1
CTSW	0	0	0	0	0	0	0	1	0	1
CD8A	0	0	1	0	0	0	0	0	0	1
CPLX1	0	0	0	0	0	0	0	0	1	1

Figure A3.1 List of genes differentially regulated genes (>5 or < -5 fold) between T-ALL and normal thymocytes with detailed number of transcription factor binding sites for each transcription factor.

APPENDIX 4

Genes which were differentially regulated (>5 or < -5 fold) and has at least a total of 10 binding sites for the 9 Ttranscription factors (163 genes obtained from CONFAC software, (APPENDIX-3) were intersected with the 4185 genes targeted by differentially expressed microRNAs to obtain a list of 64 unique genes (216 unique gene-microRNA pairs). Among 216 microRNA-gene pairs, 94 microRNA- gene pairs (61 unique genes) were present whose expression were anti-correlated.

GENE	MicroRNA	MicroRNA Sign	GENE Sign	RELATIONSHIP
EXOC5	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
NMT2	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
SLC35A3	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
AXIN2	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
BCL11A	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
LRRN3	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
RBM6	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
DACH1	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
PHF20L1	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
TAF15	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
NFATC3	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
RECK	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
PROSC	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
ACSBG1	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
GRAMD3	hsa-mir-107	POSITIVE	NEGATIVE	ANTI- CORRELATED
IRS1	hsa-mir-126	POSITIVE	NEGATIVE	ANTI- CORRELATED
DDX6	hsa-mir-132	NEGATIVE	POSITIVE	ANTI- CORRELATED
SP1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
CAMK1D	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
ABCA1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
DACH1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
FUS	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
RAG1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
C15oRf29	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
BMPR2	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
BCL11A	hsa-mir-190	POSITIVE	NEGATIVE	ANTI- CORRELATED
PHF20L1	hsa-mir-190	POSITIVE	NEGATIVE	ANTI- CORRELATED

LRRN3	hsa-mir-190	POSITIVE	NEGATIVE	ANTI- CORRELATED
WSB1	hsa-mir-190	POSITIVE	NEGATIVE	ANTI- CORRELATED
TOP1	hsa-mir-193b	NEGATIVE	POSITIVE	ANTI- CORRELATED
DDX6	hsa-mir-205	NEGATIVE	POSITIVE	ANTI- CORRELATED
B3GNT5	hsa-mir-205	NEGATIVE	POSITIVE	ANTI- CORRELATED
PLEK	hsa-mir-205	NEGATIVE	POSITIVE	ANTI- CORRELATED
TOP1	hsa-mir-205	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDK6	hsa-mir-206	NEGATIVE	POSITIVE	ANTI- CORRELATED
TAL1	hsa-mir-206	NEGATIVE	POSITIVE	ANTI- CORRELATED
PIK3C2A	hsa-mir-223	POSITIVE	NEGATIVE	ANTI- CORRELATED
SP1	hsa-mir-223	POSITIVE	NEGATIVE	ANTI- CORRELATED
PHF20L1	hsa-mir-223	POSITIVE	NEGATIVE	ANTI- CORRELATED
DDX6	hsa-mir-22	NEGATIVE	POSITIVE	ANTI- CORRELATED
CEBPD	hsa-mir-22	NEGATIVE	POSITIVE	ANTI- CORRELATED
LGALS1	hsa-mir-22	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDK6	hsa-mir-22	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDK6	hsa-mir-26a	NEGATIVE	POSITIVE	ANTI- CORRELATED
FNIP1	hsa-mir-26a	NEGATIVE	POSITIVE	ANTI- CORRELATED
ESCO1	hsa-mir-26a	NEGATIVE	POSITIVE	ANTI- CORRELATED
TOP1	hsa-mir-26a	NEGATIVE	POSITIVE	ANTI- CORRELATED
BCL11A	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
SP1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
IKZF1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
SUV420H1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
IRS1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
SMPD3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
EGR3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
CALM3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
ZNF148	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
SGPP1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
GSPT1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
SLC35A3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
ID3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
MARCKS	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
FBLN5	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
ABCA1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
WSB1	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
ADD3	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
ARNTL	hsa-mir-27a	POSITIVE	NEGATIVE	ANTI- CORRELATED
IRS1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED

STAT1	hsa-mir-145	POSITIVE	NEGATIVE	ANTI- CORRELATED
SP1	hsa-mir-150	Unknown	NEGATIVE	ANTI- CORRELATED
ZNF148	hsa-mir-150	Unknown	NEGATIVE	ANTI- CORRELATED
EGR2	hsa-mir-150	Unknown	NEGATIVE	ANTI- CORRELATED
NOTCH3	hsa-mir-150	Unknown	NEGATIVE	ANTI- CORRELATED
TCF12	hsa-mir-150	Unknown	NEGATIVE	ANTI- CORRELATED
TAL1	hsa-mir-296-5p	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDK6	hsa-mir-320a	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDC27	hsa-mir-433	NEGATIVE	POSITIVE	ANTI- CORRELATED
TOP1	hsa-mir-433	NEGATIVE	POSITIVE	ANTI- CORRELATED
TOP1	hsa-mir-549	NEGATIVE	POSITIVE	ANTI- CORRELATED
NFKBIZ	hsa-mir-601	NEGATIVE	POSITIVE	ANTI- CORRELATED
DDX6	hsa-mir-646	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDK6	hsa-mir-646	NEGATIVE	POSITIVE	ANTI- CORRELATED
LATS2	hsa-mir-646	NEGATIVE	POSITIVE	ANTI- CORRELATED
CDC27	hsa-mir-646	NEGATIVE	POSITIVE	ANTI- CORRELATED
DDX6	hsa-mir-650	NEGATIVE	POSITIVE	ANTI- CORRELATED
PRDM2	hsa-mir-708	NEGATIVE	POSITIVE	ANTI- CORRELATED

Table A4.1 Anti-correlated genes and microRNAs: 94 gene-microRNA pairs, 61 genes.
POSITIVE=Upregulated in T-ALL, NEGATIVE = Down Regulated in T-ALL.

APPENDIX 5

All the core enrichment genes (137 in number) related to different KEGG categories were collected (Appendix- 2) and intersected with 4185 genes (7430 microRNA-gene pairs) to obtain 107 microRNA-gene pairs. Among these 107 microRNA gene pairs, 42 microRNA-gene pairs expression were found to be anti-correlated.

GENE	MicroRNA
CNOT6L	hsa-mir-107
PPP2R5C	hsa-mir-107
AXIN2	hsa-mir-107
LRP6	hsa-mir-107
CD28	hsa-mir-107
NFATC3	hsa-mir-107
AKT3	hsa-mir-107
LRP6	hsa-mir-126
CNOT6L	hsa-mir-145
ABCA1	hsa-mir-145
CD28	hsa-mir-145
SMAD4	hsa-mir-145
RAG1	hsa-mir-145
XRN1	hsa-mir-145
NLGN4X	hsa-mir-190
SMAD4	hsa-mir-190
PAPOLG	hsa-mir-190
PRKACB	hsa-mir-223
PIK3C2A	hsa-mir-223
IL6ST	hsa-mir-223
DCP2	hsa-mir-27a
LRP6	hsa-mir-27a
NLGN4X	hsa-mir-27a
CALM3	hsa-mir-27a
CNOT1	hsa-mir-27a
ICOS	hsa-mir-27a
PDK1	hsa-mir-27a

CD28	hsa-mir-27a
STAG1	hsa-mir-27a
YWHAB	hsa-mir-27a
ABCA1	hsa-mir-27a
CREB1	hsa-mir-27a
FYN	hsa-mir-27a
STAT1	hsa-mir-145
AKT3	hsa-mir-150
CNOT6L	hsa-mir-320a
TBL1XR1	hsa-mir-320a
CALM3	hsa-mir-320a
SPRED2	hsa-mir-320a
LRP6	hsa-mir-320a
CNOT4	hsa-mir-320a
STAG2	hsa-mir-320a

Table A5.1 List of 42 core enriched anti-correlated gene-microRNA pairs.

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