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Role of 3’UTR Elements in the Regulation of the Cyclin D1 Proto-oncogene

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To

Aai and Baba
who have continuously showered me with love
and affection…

Abbreviations

Ago	Argonaut
ALCL	Anaplastic large cell lymphoma
AREs	AU-rich elements
ATM	ataxia talengiectasia mutated
AUBP	ARE binding protein
B-ALL	B acute lymphoid leukemia
CCND1	Cyclin D1
CDK	Cyclin dependent kinase
CLL	Chronic lymphocytic leukemia
del	Deletion
Fw	Forward
GFP	Green fluorescent protein
IL	Interleukin
ins	Insertion
MCL	Mantle cell lymphoma
miRNAs	MicroRNAs
mRNA	Messenger RNA
NFκB	Nuclear factor κB
NHL	non-Hodgkin's lymphoma
nt	Nucleotide
PCR	Polymerase chain reaction
PTEN	phosphotase and tensin homologue
Rb	Retinoblastoma
Rev	Reverse
RISC	RNA-induced silencing complex
T-ALL	T acute lymphoid leukemia
TBP	TATA-box binding protein
UTR	Untranslated region
YFP	Yellow fluorescent protein

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Introduction

1.1 Oncogenesis:

Cancer is now widely viewed as a multistep process involving the progressive accumulation of multiple mutations leading to the activation of oncogenes and the inactivation of tumor suppressor genes. Often, the deregulation of distinct pathways and processes by these accumulating mutations is a necessary prerequisite for tumor formation. Recent studies involving genetic and molecular techniques have provided tremendous insights into the biology of lymphoma. These studies have led to the understanding that lymphoma is composed of several distinct subsets with characteristic genetic, cytogenetic, biochemical and morphological features. The recent focus in lymphoma research has been the identification and characterization of specific molecular alterations and the determination of their correlation with disease progression and treatment outcome. Genetic changes in lymphoma include microscopically detectable chromosomal alterations, sub-microscopic alterations such as small deletions and inversions and even more inconspicuous mutations at the genomic level.

Since the discovery of the Philadelphia chromosome, recurrent chromosomal abnormalities such as translocations, deletions, inversions and duplications associated with several types of leukemia, lymphoma as well as certain types of epithelial tumors have been identified (Look, 1997; Rabbitts, 1994; Rowley, 1998). These chromosomal abnormalities are often somatic mutations acquired by a clonally expanded malignant population. As is the case with CML, certain chromosomal abnormalities can be associated with specific types of disease and the characterization of these abnormalities can be used for diagnosis as well as for the determination of disease prognosis. Moreover, treatment regimens can be optimized to suit discrete sub-groups divided according to these abnormalities.

Chromosomal abnormalities such as translocations cause oncogenic activation through one of two mechanisms. The first mechanism involves a fusion of the coding regions of two proto-oncogenes, which in turn results in a chimeric or fusion protein with altered functions. As an example, the t(15;17) translocation fuses the retinoic acid receptor α (RAR α) to the myeloid

transcription factor PML, forming a fusion product in acute promyelocytic leukaemia (APL). (Kakizuka et al., 1991). The second mechanism involves the juxtaposition of proto-oncogenes to one of the regulatory regions of immunoglobulin genes or T-cell receptor loci (Boehm and Rabbitts, 1989; Cleary, 1991; Korsmeyer, 1992). These latter type of rearrangements are mostly observed in lymphoid malignancies such a B and T acute lymphoid leukemia (B-ALL and T-ALL) and different types of lymphomas. Examples of this type include the t(8;14) in Burkitt lymphoma/leukemia and the t(14;18) in follicular lymphoma. The former type of chromosomal translocations occur in some types of non-Hodgkin lymphoma, such as the t(2;5) in anaplastic large cell lymphoma (ALCL), but are best known in acute and chronic myeloid leukemias.

In Mantle cell lymphoma (MCL), which will be described in more detail in the following section, the *cyclin D1* gene which is normally not expressed in normal B cells, is juxtaposed to the immunoglobulin heavy chain enhancer (IgH),by the t(11;14) translocation leading to its ectopic, constitutive overexpression .

1.2 Mantle cell lymphoma:

Mantle cell lymphoma was first described as centrocytic lymphoma, an independent subtype of non-Hodgkin's lymphoma (NHL), according to the Kiel classification in 1974. With the introduction of the general WHO tumour classification in the year 2001 this subtype was designated as mantle cell lymphoma. MCL is an aggressive form of lymphoma that accounts for about 5 to 10% of NHL. It has a poor prognosis with the average survival rate of 3-4 years. As discussed above, the t(11;14)(q13;q32) translocation is the hallmark of the disease which juxtaposes the cyclin D1 gene at 11q13 to the IgH at 14q32 leading to overexpression of cyclin D1 (Rimokh et al., 1994b).

1.2.1 Clinical characteristics of mantle cell lymphoma:

MCL is a well defined lymphoid neoplasm that is characterised by the proliferation of mature, naive B cells. These cells possess a tendency to disseminate throughout the body infiltrating the bone marrow, peripheral blood and extranodal sites. This tumor is prevalent in males with advanced age of around 60 years. Despite new therapies being developed, MCL

patients show a median survival of 3-5 years and frequent relapses. MCL has a spectrum of morphologically different cytological variants such as classical MCL, blastoid MCL and pleomorphic MCL as shown in **Fig.1.2a**

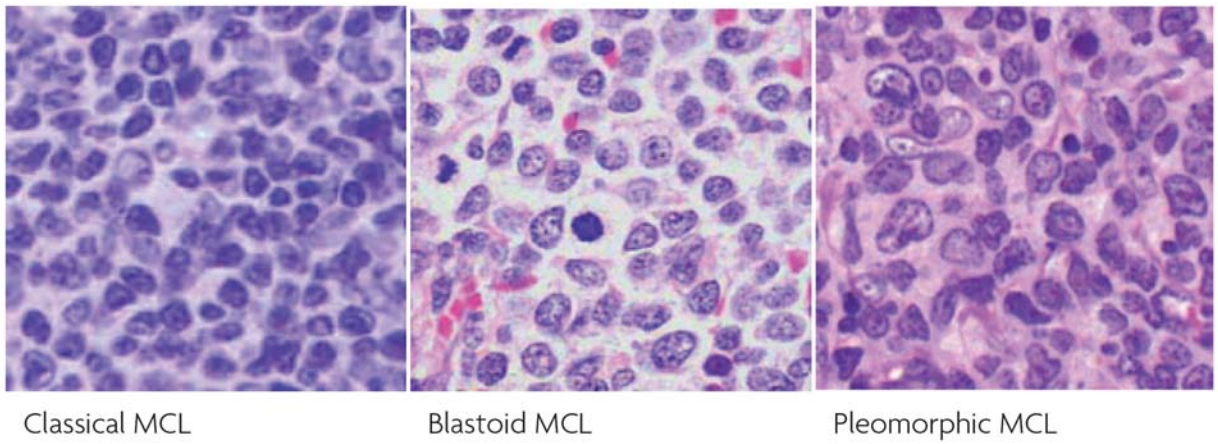


Fig.1.2a Histological variants of MCL (figure from Jares P., 2007) Different variants of MCL are depicted in the figure: classical MCL have small to medium sized B cells with irregular nuclei and inconspicuous nucleoli whereas blastoid variants show rounded nuclei, finely dispersed chromatin. The pleomorphic and blastoid variants have higher proliferation rates, complex karyotype and aggressive clinical behaviour.

1.2.2 Treatment strategies for mantle cell lymphoma:

Since various important signalling pathways are activated in MCL cells leading to enhanced cell proliferation, the treatment of MCL patients remains a challenge. Conventional therapy includes chemotherapeutic regimens based on the CHOP regimen that includes cyclophosphamide, hydroxydoxorubicin, oncovin (vincristine) and prednisone or HyperCVAD (an intense standard chemotherapeutic regimen based on the CHOP combination but also including high doses of methotrexate and cytarabine) in the initial phase of the disease. HyperCVAD is also given in combination with rituximab (anti-CD20 monoclonal antibody) for initial treatment (Jares et al., 2007; Witzig et al., 2005). However, despite the benefits of this multimodal approach, most patients relapse even after high-dose therapy. It has been stated that the only curative approach is allogeneic stem cell transplantation, which may be adapted to the

elderly MCL patient cohort by modified dose-reduced conditioning regimens (Hiddemann and Dreyling, 2003).

In past few years new strategies are being developed that target the crucial pathways and may change the management and outcome of MCL patients. As the PI-3K/mTOR pathway seems to be involved in MCL, the mTOR inhibitor Ramapamycin and its derivative RAD001 were tested in MCL cell lines. These molecules have been shown to induce cell cycle arrest without causing apoptosis. As a single agent, RAD001 inhibited proliferation in MCL cell lines. Furthermore, RAD001 showed predominantly synergistic cytotoxicity in combination with several secondary agents, including doxorubicin, vincristine or rituximab (components of the standard MCL regimen), as well as paclitaxel, vorinostat and bortezomib (Haritunians et al., 2007). These results suggest the potential use of these cytostatic drugs alone or in combination in the treatment of MCL. The other cell cycle inhibitor molecules include the CDK inhibitor Flavopiridol which is being explored in relapsed MCL patients.

The ubiquitin-proteasome system has become a promising novel molecular target in cancer due to its critical role in cellular protein degradation, its interaction with cell cycle and apoptosis regulation and its unique mechanism of action. Bortezomib as first-in-class proteasome inhibitor has proven to be highly effective in some hematological malignancies including relapsed mantle cell lymphoma (Kane et al., 2007). It reversibly inhibits the 26S proteasome, a large protein complex that degrades many of the intracellular proteins. Inhibition of the proteasome prevents this targeted proteolysis of pro-apoptotic proteins which may result in alteration of many signalling cascades in cells. In addition to all the therapies targeting different pathways, there are certain therapies used in clinical practice which induce apoptosis in tumor cells. Apoptosis depends on the activation of caspases by either intrinsic or extrinsic pathways that require activation of the tumor necrosis factor (TNF) family of cell death receptors by their related ligands and TNF-related apoptosis inducing ligand (TRAIL). TRAIL was reported to induce apoptosis selectively in neoplastic cells through DR4 receptors (MacFarlane et al., 2005). Thus, agents such as TRAIL and anti-CD4 antibodies are being investigated in the treatment of MCL.

1.2.3 Cellular biology of MCL:

The ectopic expression of *cyclin D1* in B lymphocytes is considered to be the primary event in the pathogenesis of MCL, probably facilitating the deregulation of cell cycle at G1-S transition. Analysis of the breakpoint regions in the two chromosomes has suggested that this translocation occurs in the bone marrow in an early B cell at the pre-B stage of differentiation when the cell is initiating the Ig gene rearrangement with the recombination of the V(D)J segments (Jares et al., 2007). The immunophenotype of MCL cells reflects a mature B-cell arrangement ($CD45^+$, $CD19^+$, $CD20^+$, $CD22^+$, $CD24^+$, CD $79a^+$). MCL cells are $CD5^+$and $CD43^+$ but $CD23^-$ as well as $CD10^-$. These neoplastic cells of MCL tend to colonize the mantle zone of the lymphoid follicles, hence the term mantle cell lymphoma was designated for this tumour. Although the t(11;14) translocation occurs in an immature B cell in the bone marrow, the selective oncogenic advantage of this chromosomal aberration fully develops when these cells attain the differentiation phase of a mature naive pre-germinal centre B cell (Banks et al., 1992). According to the origin from pre-germinal centre cells, most of the MCL samples have no or very few somatic mutations in V-gene sequences of Ig (V_H) genes. However, 15-40% of MCLs do carry somatic hyper-mutations which indicate that some tumors originate in cells that have undergone the influence of the mutational machinery of the follicular germinal centre.

1.2.4 Molecular biology of MCL:

Ectopic and deregulated expression of *cyclin D1* is believed to be one of the causes of oncogenesis in MCL. Cyclin D1 in combination with cyclin dependent kinases (CDK) 4 and 6 (CDK 4 and CDK 6) regulates the cell cycle transition between the G1 to the S phase. Cyclin D1 binds to the CDK 4/6, cyclin D1-CDK 4/6 complexes which then phosphorylate the retinoblastoma (Rb) protein, leading to the inactivation of its suppressive effect on cell cycle progression. The hyperphosphorylated Rb protein in turn releases the E2F transcription factors which force the cell to enter the S phase and eventually commit itself to the cell cycle. The hyperphosphorylation of Rb in MCL cases demonstrates the role of cyclin D1 in these tumors by overcoming the cell cycle suppressive effect of Rb (Jares et al., 1996). However, recently identified intragenic deletions of Rb leading to complete lack of protein expression in some cases of MCL suggest an oncogenic role of cyclin D1 independent of Rb in these tumors (Pinyol et al.,

2007). Overexpressed cyclin D1 sequesters free $p27^{kip1}$ into cyclin D1-CDK4/6 complexes which would decrease the active form of $p27^{kip1}$ in the cell, making it incapable of inducing G1 cell cycle arrest. This inactive form of $p27^{kip1}$ might be reducing inhibition on CDK2-cyclin E, facilitating entry into S phase thus potentiating lymphomagenesis (Quintanilla-Martinez et al., 2003).

Apart from cyclin D1 overexpression, there are other dysregulated pathways which contribute to pathogenesis of MCL. Cytogenetic analysis has demonstrated that MCL is one of the lymphoid neoplasms with the highest number of chromosomal aberrations that include losses, gains and amplification of genes involved in tumor progression (Salaverria et al., 2006). Clinical observations also supported the need for additional oncogenic events in MCL. The search for potential target genes altered in MCL has identified that most of them are involved in two common pathogenetic pathways, cell cycle and the DNA damage response **(Fig.1.2b)**.

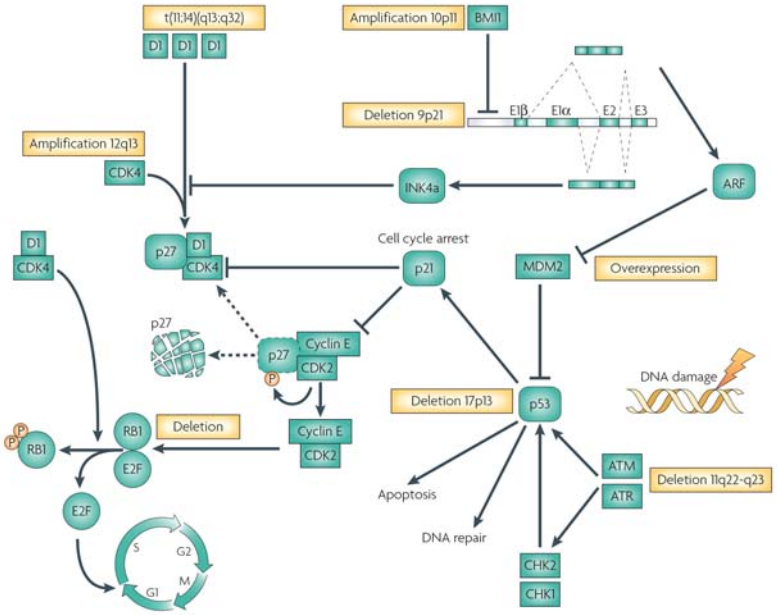


Fig.1.2b Cell cycle and DNA damage response pathway dysregulation in MCL. (Figure from Jares P. et al., 2007) This figure depicts the involvement of different genes in deregulation of the cell cycle and DNA damage response pathway, cell cycle deregulation is indicated on the left hand side of the figure whereas the right hand side shows the factors involved in the DNA damage response pathway.

Cell cycle deregulation: The two main regulatory pathways involved in the control of cell cycle and senescence, namely the INK4a-CDK4-RB1 and the ARF-MDM2-p53 pathways are dysregulated in highly proliferative MCLs. Inactivation of CDKN2A is observed in a large number of cases. The locus on chromosome 9p21 which is deleted in MCLs, encodes two key regulatory elements, CDK inhibitor INK4a and the p53 regulator ARF. As the name suggests INK4a inhibits CDK4 and 6 and maintains Rb in its active form. Thus, the genomic deletion of 9p21 and cyclin D1 overexpression, together lead to the hyperphosphorylation of Rb and cell cycle progression. The prime function of ARF (located at 9p21) is to protect p53 from degradation by MDM2 mediated proteasome inhibition (Rosenwald et al., 2003). **(Fig.1.2b)**. p53 inactivation is therefore caused by high levels of MDM2 and deletion of 9p21 locus in some of MCL cases (Hernandez et al., 2005). Tumors with wild type CDKN2A show mutated p53 and CDK4 amplification which suggests that the simultaneous inactivation of both pathways, INK4a-CDK4-RB1 and ARF-MDM2-p53 probably provides a selective proliferative advantage to the cells.

DNA damage response pathway alterations: The ataxia talengiectasia mutated (ATM) gene encodes a serine-threonine kinase belonging to the phosphotidylinositol-3 kinase (PI-3K) family. This enzyme is involved in DNA damage response signalling pathways. Different studies have now identified a number of targets of the ATM gene including p53. In response to various DNA damaging agents, ATM phosphorylates p53 leading to its stabilization and cell cycle arrest and apoptosis. This ATM gene is mutated in different lymphoproliferative diseases including MCL (Camacho et al., 2002). These mutations mainly affect the PI-3K domain or lead to truncated *ATM* proteins that are rapidly degraded. Due to ATM mutation and p53 inactivation, there is loss of apoptosis which is one of the mechanisms of MCL pathogenesis.

Cell survival pathway dysregulation: In addition to the alterations in cell cycle control and DNA damage response pathways; MCL may also have activated cell survival mechanisms. Recent gene profiling studies showed that many individual genes involved in signalling pathways that affect cellular proliferation are either overexpressed or underexpressed in MCL cases. Many of these overexpressed genes are the members of PI-3K Akt signalling pathway including *PIK3CA, Akt-1, PDK-1,* and *PPP1R2*. This signaling pathway is known to be involved in the transduction of extracellular stimuli that regulate fundamental cellular processes including cell-

cycle progression, proliferation and cell growth, apoptosis, and survival (Rudelius et al., 2006). This pathway is reported to be constitutively active in a subset of MCL that includes the aggressive blastoid variants and is associated with the loss of its negative regulator, phosphatase and tensin homologue (PTEN). Further, constitutive activation of nuclear factor κB (NFκB), that regulates genes involved in both cell survival as well as apoptotic signalling pathways has also been reported in MCL cell lines and primary tumours.

Thus, the activation of these two major pathways leads to increased cell proliferation, survival and inhibition of apoptosis in MCL.

1.3 Cyclin D 1:

The cell cycle clock coordinates the progression of eukaryotic cells through their growth and division cycles. Its importance is being increasingly appreciated as it is the master controller of a cell's decision to continue proliferating or to withdraw from the cycle and enter a state of quiescence. In 1998, Robert Weinberg's group (Jacks and Weinberg, 1998) pointed to a wider role for components of the cell cycle machinery. The core components of it include cyclins and cyclin dependent kinases along with cyclin dependent kinase inhibitors, Rb protein (Jacks and Weinberg, 1998). As the name cyclins suggests, the amounts of these proteins oscillate during different phases of cell cycle. There are three types of cyclins in mammalian cells, S phase cyclins, G2-M cyclins and G1 cyclins. G1 cyclins include D and E type cyclins. D type cyclins are cyclin D1, D2 and D3. The cyclin D1 protein plays an important role in the G1-S transition of a cell.

The human cyclin D1 gene was initially cloned as a breakpoint rearrangement in parathyroid adenoma. Motokura and colleagues found the D11S287E transcript that is always overexpressed in parathyroid adenomas, and named it PRAD1 (Motokura et al., 1991). Other researchers later identified it as cyclin D1 (CCND1). Cyclin D1 encodes two distinct mRNA transcripts, a 1.7kb transcript, (transcript b) and a longer 4.4kb (transcript a) (Betticher et al., 1995). Sequencing of the cyclin D1 mRNA from these two transcripts shows no differences in the coding region but a single base pair A/G polymorphism at position nt870. Earlier it was thought that this polymorphism plays a role in alternative splicing by favoring the expression of

one of the two isoforms. It was shown that in case of the 'G' allele, splicing from exon 4 to exon 5 (transcript a) is favoured and the 'A' allele is more likely not to splice at this junction leaving the coding sequence reading into intron 4. The protein from transcript b (isoform b) differs in the carboxy-terminus from isoform a, which bears the PEST destruction box. Phosphorylation of cyclin D1 takes place at a conserved carboxy terminal residue Thr 286, which exports it out of the nucleus at the G1-S boundary. Since isoform b lacks this carboxy-terminus, it has been shown to be an isoform that always remains in the nucleus during the cell cycle unlike the canonical isoform a (Betticher et al., 1995).

1.3.1 Cyclin D1 as cell cycle regulator:

As mentioned above, cyclin D1 has been reported to be one of the key regulators of cell cycle machinery especially in G1 to S transition. It serves as a key sensor and integrator of extracellular signals of cells in early to mid-G1 phase mediating its function through binding both the CDKs and histone acetylases and deacetylases to modulate local chromatin structure of genes that are involved in cell proliferation and differentiation (Huang et al., 1998). Cyclin D1 encodes the regulatory subunit of the serine-threonin kinases (CDKs), CDK4 and CDK6. The identification of the catalytic partners of the D-type cyclins established these proteins as bona fide cyclins. Cyclin D1 binds to CDK4/6 to form cyclin/CDK complexes. These complexes further act on Rb and cause its phosphorylation. Rb acts as a gatekeeper at the G1-S transition by binding to the E2F transcription factors. Phosphorylation of Rb at the C terminus due to cyclin/CDK complexes inhibits its function of restricting the entry of a cell into the S phase. This promotes the release of E2F transcription factors from Rb-E2F complexes. This in turn leads to transcriptional induction of genes necessary for progression from G1 to S phase; most notably cyclin E. Complete inactivation of Rb is mediated by the cyclin E-CDK2 holoenzyme, whose activation is facilitated by non-catalytic function of the cyclin D-CDK complex involving the titration of CDK inhibitors, p21 and p27. Thus, cell cycle regulation is a CDK dependent function of cyclin D1.

1.3.2 CDK independent functions of cyclin D1:

In addition to a well established role in controlling the progression of cell through G1-S phase in cell cycle in concert with CDKs, CKD independent roles for cyclin D1 have also been

documented. Cyclin D1 has been shown to affect the activity of several transcription factors. It forms a physiological association with 30 different transcription factors or coregulators including androgen receptor (AR), estrogen receptor (ER) α, thyroid hormone receptor and STAT3 (signal transducer and activator of transcription) (Bienvenu et al., 2001; Coqueret, 2002; Knudsen et al., 1999; Neuman et al., 1997). It has been reported that cyclin D1 associates with the TATA-box binding protein associated factor(II)250 and suppresses pRb mediated inhibition of TATA-box binding protein associated factor(II)250 kinase activity (Siegert et al., 2000) . In addition to *in-vitro* analysis of cyclin D1 functions, mouse models have also been studied to demonstrate the physiological role for cyclin D1. Mice deficient in cyclin D1 are small and display retinal hypoplasia and defects in lobulo-alveolar development during pregnancy. Creating mice deficient both in cyclin D1 and p27 rescued the defects that characterize cyclin D1 deficient mice which prove the aforementioned physiological role for cyclin D1 (Ewen and Lamb, 2004; Fantl et al., 1995).

1.3.3 Cyclin D1 as proto-oncogene:

In the mammalian cell cycle, there is a restriction point at the entry of S phase. This point was defined for the first time, as the time point at which the cell loses reliance upon external signals necessary for continued cell cycle progression. The restriction point control is proposed to permit normal cells to retain viability by a shift to a minimal metabolism upon differentiation *in-vivo* and *in-vitro* when conditions are suboptimal for growth. Malignant cells are believed to have lost their restriction point control and this loss is described as the consequence of overexpressed cyclin D1 (Pardee, 1974). Uncontrolled cell proliferation is the hallmark of cancer and tumor cells typically acquire mutations of genes that directly regulate their cell cycles. It has been proposed that oncogenic processes exert their greatest effect by targeting regulators of G1 phase progression in particular (Sherr, 1996). The pRb and p53 pathways have been observed to be dysregulated in most cancer types and cyclin D1 is targeted by both of these pathways. Its overexpression in several different malignancies, including breast cancer, parathyroid cancer, colon cancer and mantle cell lymphoma is believed to lead to increased cell proliferation and tumor formation.

Deregulation of cyclin D1 in cancer

Amplification, mutation or overexpression may be the cause of deregulated cyclin D1 in various neoplasms. Callender et al. reported the amplification of cyclin D1 gene in around 34% of head and neck squamous carcinoma samples (Callender et al., 1994). Amplification was more noted in high grade, high stage, and aneuploid tumors showing a highly statistical correlation between PRAD-1 amplification and proliferative activity. This indicated that cyclin D1 amplification is a late event in the tumorigenesis of head and neck carcinoma and is often associated with a subset of aggressive tumors and highly proliferative neoplasms. Amplification of the cyclin D1 gene was also observed in breast cancer cell lines and its overexpression was observed in 45% of primary tumors (Buckley et al., 1993). Similarly, cyclin D1 amplification was observed in neuroblastoma tumors and cell lines (Molenaar et al., 2003). In some tumors, the degree of overexpression of this proto-oncogene is said to be higher than what can be attributed to cyclin D1 gene amplification. Cyclin D1 amplification was found in 15% of primary human breast cancers (Hosokawa and Arnold, 1998) and overexpression in 30-50% of breast cancers, suggesting that mechanisms in addition to DNA amplification may lead to deregulated expression of this gene in breast cancer.

As mentioned previously, proto-oncogene overexpression can result from genomic translocations involving the oncogene. The cyclin D1 gene was discovered as a transcript on chromosome 11q13 (initially termed D11S287E) which is clonally rearranged with the parathyroid hormone locus in a subset of benign parathyroid tumors. In parathyroid adenomas and carcinomas, the cyclin D1 protein is overexpressed as a result of its clonal rearrangement, leading to tumorigenesis (Rimokh et al., 1994b). In MCL, the t(11;14)(q13;q32) translocation juxtaposes the cyclin D1 gene to the IgH enhancer which leads to overexpression of the protein contributing to tumor formation. Recently, other mechanisms including mutations in cyclin D1 at the genomic level have been reported in addition to the translocation which might play a role in overexpression of cyclin D1 in MCL.

Even though mutations that alter the coding sequence of this gene have rarely been reported in cancer, there are several reports that point to somatic mutations in the 3' untranslated region (UTR) of the cyclin D1 mRNA. A truncation of the cyclin D1 gene in its 3'UTR has been reported in a human breast cancer cell line associated with overexpression of the protein. In

mantle cell lymphoma patients, a truncation in 3'UTR of cyclin D1 gene was observed and it was proposed that the truncation of the 3'UTR eliminates cis-acting elements such as AU rich sequences (AREs) leading to increased mRNA stability and hence increase in cyclin D1 protein expression (Rimokh et al., 1994a). In addition to these mutations, the *cyclin D1* isoform b which harbors a shorter 3'UTR has been reported to be the cause of tumorigenesis in cancers such as esophageal carcinomas (Lu et al., 2003). People have suggested that the isoform b being nuclear during the cell cycle binds to CDK4/6 and constitutively inactivates the pRb pathway leading to increased cell proliferation and tumorigenesis in different cancers including MCL. However, it has recently been proved that both the isoforms exist in all tumor samples as well as in their normal counterparts and the ratio of mRNA expression of isoforms a and b does not play any role in cancer progression (Krieger et al., 2006; Wiestner et al., 2007). The *cyclin D1* isoform b, although present in the nucleus, can not inactivate the pRb pathway, thereby causing no changes in cell cycle progression. Increased stability of isoform b was also predicted to be the cause of cyclin D1 mediated tumorigenesis but it was recently shown to have comparable stability as compared to the a isoform (Holley et al., 2005; Leveque et al., 2007; Solomon et al., 2003). In fact the shorter isoforms of the cyclin D1 gene in MCL result from genomic deletions of the 3'UTR or from point mutations that prematurely truncate the *cyclin D1* 3'UTR in a majority of MCL patients. These shorter mRNAs are *cyclin D1* a isoforms, not alternatively spliced *cyclin D1b* mRNA isoforms (Wiestner et al., 2007). Premature polyadenylation due to a 3'UTR mutation is also observed in the Z-138 MCL cell line expressing both truncated and full-length *cyclin D1a* mRNAs. Mutations in the 3'UTR create the premature polyadenylation signals within 300 bp from the stop codon. A 3 bp 'TCA' deletion at 309-311 position after the stop codon of cyclin D1 is found in one MCL case whereas a single bp 'A' insertion between nucleotides number 308 and 309 in another. Both types of mutations create a polyadenylation signal at an identical site, 304 bp 3' of the stop codon. Interestingly, shorter mRNAs of *cyclin D1* are more stable than full-length mRNAs since they lack mRNA destabilizing elements as discussed by Rimokh et al. These data thus suggest that genetic events causing mutations or deletion in the 3'UTR of the cyclin D1 gene enhance the effect of the t(11;14) translocation by generating higher levels of *cyclin D1* mRNAs and protein, leading to an increased cell cycle progression through the G1-S phase. These observations made in MCL and other tumours point to the role of 3'UTR of cyclin D1 gene in malignant transformation. Detailed studies on the contribution of this region

to *cyclin D1* expression and to malignant transformation are therefore necessary and are the focus of this dissertation.

1.4 3'UTR mediated gene regulation:

Eukaryotic gene expression is controlled at the levels of transcriptional and translational initiation and elongation at both these levels regulates gene expression. After transcription, the messenger RNA (mRNA) is either translated into protein or degraded, depending upon its function in the cell. mRNAs for genes such as *c-fos,* that is involved in the response of cells to the external environment, are degraded rapidly in the cytoplasm with a half life of 8-30 minutes whereas *β-globin* mRNA in erythroid cells is quite stable with a half life of greater than 24 hours (Atwater et al., 1990). Cyclins, genes involved in cell cycle regulation, undergo rapid turnover with less mRNA stability. The stability of mRNA depends on different regions like the 3'and 5'UTRs containing *cis*-acting elements and *trans*-regulating factors binding directly or indirectly to these *cis*-acting elements. The formation of mRNA-protein complexes followed by a series of re-modelling events, influence the translation and decay of mRNAs.

The 3'UTR of mRNAs is proposed to be one of the most important factors regulating mRNA stability. The presence of numerous *cis*-acting elements in this region and the binding of proteins, make it an important regulatory unit. It has been shown that alterations in the 3'UTR of many transiently expressed genes leads to enhanced stability of their mRNAs leading to overexpression and oncogenesis (Jones and Cole, 1987; Mayr et al., 2007) indicating its role in gene expression. There are several recognised motifs in 3'UTRs that could be crucial for the regulation of mRNAs. Of these, AU-rich elements (AREs) have been characterised in some detail. Moreover, in the last few years, there is mounting evidence that the 3'UTR of several mRNAs harbour binding sites for microRNAs, a class of small noncoding RNAs that regulate gene expression. Since this thesis describes the regulation of the cyclin D1 gene by these two types of motifs in the 3'UTR, AREs and microRNAs will be discussed in the following sections.

1.4.1 AU rich elements in the 3'UTR:

Adenylate/uridylate rich elements or AREs are the highly conserved elements found in the 3'UTRs of many mRNAs that code for proto-oncogenes, cytokines and nuclear transcription factors (Chen and Shyu, 1995) and function as elements that are important for mRNA stability. They are the most common and well studied *cis*-acting elements in the 3'UTR. 10% of human mRNAs are reported to contain AREs in their 3'UTRs. The first direct evidence that the ARE can function as a potent mRNA destabilizing element came from a study by Shaw and Kamen in 1986. The stability of β-globin mRNA was reduced considerably by inserting a conserved region of 51 nucleotides containing AUUUA motifs from the 3'UTR of human granulocyte-macrophage colony-stimulating factor (GM-CSF) into its 3'UTR (Shaw and Kamen, 1986). Later, the mRNA degradation of *c-fos*, *c-myc*, β-interferon and many more genes was also shown to be the effect of AREs in their respective 3'UTRs (Chen et al., 1994; Chen and Shyu, 1994; Jones and Cole, 1987; Peppel et al., 1991).

AREs are proposed to be of three types, class I and II AREs containing various repeats of AUUUA signature motifs and class III lacking this pentamer (Xu et al., 1997). There appears to be a sequence hierarchy by which the three classes of AREs are composed from several key sequence features. AREs classified as class I are found in early-response gene mRNAs that encode various transcription factors and mRNAs for some cytokines like IL-4, IL-6. They contain one to three copies of AUUUA motifs coupled with nearby U-rich sequences. Each of these sequence features plays a distinct role and together they determine the destabilizing potency of the class I AREs (Chen et al., 1994). Class II AREs possess multiple repeats of the AUUUA pentamer clustered together and the class III AREs contain only a couple of U stretches and a U-rich domain. The class II AREs direct asynchronous cytoplasmic deadenylation which is consistent with a processive ribonucleolytic digestion of polyA tails whereas class I and AUUUA less class III AREs mediate synchronous polyA shortening followed by the decay of the mRNA. Existence of cross talk between the AREs and the 3' end polyA tail dictates the rate and kinetics of cytoplasmic deadenylation and thereby the fate of the corresponding mRNA (Xu et al., 1997). The length of AREs, especially the number of overlapping pentamers may also contribute to mRNA half life (Akashi et al., 1994). When ARE mRNAs in endotoxin stimulated monocyctes

were clustered into class I and class II categories by bioinformatic analysis, the mRNAs having class II AREs showed shorter half lives than those of class I (Frevel et al., 2003; Khabar, 2005).

Mechanisms of ARE mediated mRNA stability:

As mentioned above, AREs play a major role in mRNA stability by either degradation or enhanced translation. One of the mechanisms of ARE mediated mRNA degradation or stabilization includes binding of ARE binding proteins (AUBP) to ARE mRNAs. HuR and AUF1 are among the AUBPs that have been discovered. HuR, a member of the ELAV/Hu family consists of the closely related proteins,HuB, HuC, and HuD (Ma et al., 1996). It has been shown to bind and stabilize many ARE mRNAs in several different cell systems. It is mainly located in the nucleus but shuttles between cytoplasm and nucleus depending on the stabilization of mRNA transcripts. Unlike HuR, AUF1 is one of the mRNA destabilizing AUBPs. The *AUF1* gene is expressed as four isoforms designated by their molecular weights, p37, p40, p42 and p45 which are generated by alternative splicing of a pre-mRNA transcript (Wagner et al., 1998). All four isoforms are predominantly nuclear but their transport between nucleus and cytoplasm is dependent on external stimuli (David et al., 2007; Sarkar et al., 2003; Suzuki et al., 2005). A structurally different class of proteins, the CCCH tandem zinc finger family also includes some AUBPs such as tristetraproline (TTP). It has been reported to bind to and stabilize several mRNAs such as *IL-3*, *TNF-α* and *GM-CSF* (Blackshear, 2002; Carballo et al., 2000; Stoecklin et al., 2000). The AUBPs may not degrade or stabilize the mRNAs directly but may be through complex protein-protein interactions. They may also regulate the mRNA stability through their interaction with the exosome, a multiprotein complex that has 3'-5' exonuclease activity (Chen et al., 2001). In addition to these AUBPs, the ubiquitin-proteasome pathway has also been shown to participate in ARE mediated mRNA decay. The link between the proteasome pathway and rapid degradation of cytokine mRNAs has been reported suggesting a complex interplay between proteasomes, polyA binding protein (PABP), heat shock proteins and translational control in ARE-mRNA decay (Laroia et al., 1999).

Some signalling pathways have also been shown to be involved in ARE mediated mRNA stability. The p38 mitogen-activated protein kinase (MAPK) pathway has been reported to play a role in degradation of different mRNAs such as *IL-3*, *IL-6*, *TNF-α* and *VEGF* (Pages et al., 2000; Wang et al., 1999; Winzen et al., 1999). The AU rich 3'UTRs of IL-6 and TNF- α are

downstream of p38 MAP kinase-activated protein kinase (MAPKAP K2 or MK2) and make it an essential component of their ARE dependent mRNA stabilization. It is proposed that the MK2 phosphorylates an uncharacterised downstream target that directly or indirectly binds to AREs leading to ARE mediated mRNA translational control.

Thus, by different mechanisms, AREs play a role in 3'UTR mediated post transcriptional regulation of numerous genes. The deletion of these elements could contribute to pathogenesis in certain disorders including cancer. As an example, in systemic lupus erythematosus (SLE) T-cells, there is reduced expression of TCR ζ protein which is the result of alternatively spliced isoform of TCR ζ that undergoes a deletion within the 3'UTR. This deletion includes two repeats of the ARE pentamer (Chowdhury et al., 2006). The reduced protein expression is linked to the deletion of AREs in the 3'UTR that otherwise either stabilize the TCR ζ mRNA or increase its translation. In addition, the transforming ability of the oncogene c-fos was shown to correlate with the deletion of an ARE containing region from its 3'UTR. (Raymond et al., 1989).

1.4.2 MicroRNAs (miRNAs):

1.4.2.1 The discovery:

In 1981 Chalfie M. et al. discovered the *Caenorhabditis elegans* lin-4 gene (Chalfie et al., 1981) which was found to play an important role in larval development since mutations in this gene caused heterochronic defects. Later in 1993, it was found that lin-4 does not encode a protein (Lee et al., 1993). Two small lin-4 transcripts of approximately 22nt and 61nt were identified in *C.elegans*, the 61nt long transcript was predicted to form a hairpin secondary structure and is the precursor of the 22nt RNA. Lin-4 was found to contain sequences complementary to a repeated sequence element in the 3'UTR of lin-14 mRNA suggesting that lin-4 regulates the translation of lin-14 by antisense RNA-RNA interaction. Further it was reported that lin-28, a cold-shock protein that regulates the transition between larval stages 2 and 3, also possesses the sequences complementary to the 22 nt transcript of lin-4 in its 3'UTR (Moss et al., 1997). Thus, the lin-4 gene was found to regulate the protein expression of the mRNAs, lin-14 and lin-28. Later Reinhart and colleagues discovered let-7, (Reinhart et al., 2000) and reported that the let-7 RNA regulates developmental stages in *C.elegans*. For some time lin-4 and let-7 were the only known naturally occurring small antisense RNAs in eucaryotes. Later

homologues of let-7 were found in D.melanogaster, animals and humans. Studies in plants and fungi uncovered the mechanisms by which these transgenes regulate homologous genes (Pal-Bhadra et al., 1997). This mechanism was described as cosuppression or posttranscriptional gene silencing (PTGS).

PTGS was followed by the discovery of dsRNA which worked better than the antisense-RNA mediated gene silencing. Hence, this finding was fundamental for the identification of dsRNA as the trigger for PTGS. Soon after, the term RNA interference (RNAi) was coined as one of the cellular mechanisms conserved among most eucaryotes. Further discoveries showed the connection between small regulatory RNAs and RNAi. It was shown that dsRNA is specifically processed to 21nt RNA in Drosophila embryonic extract. These RNAs were named as small interfering RNAs (siRNAs) (Elbashir et al., 2001) which guide the RNAi machinery to target mRNAs. The RNAi mechanism, specifically siRNAs seemed reminiscent of lin-4 and let-7 RNAs, fostering the idea that these regulatory RNAs may be natural RNAi triggers (Chang and Mendell, 2007). Further the presence of let-7 was reported in human cell lines and other human and Drosophila RNAs with conserved stem-loop precursors were discovered which were collectively named as microRNAs (miRNAs). After the discovery of the first few hundred miRNAs by cloning, sufficient miRNA sequences were available to begin applying bioinformatic analyses to scan genomes for additional miRNA genes. Thus over the past few years, the field of miRNAs has been extensively explored since these small non-coding RNAs have been shown to fine tune the expression of several genes.

1.4.2.2 Biogenesis of miRNAs:

MiRNAs are 20-22nt long, transcribed from long autonomous transcription units. Some of the miRNAs that were found in close proximity to others were found to be clustered miRNAs

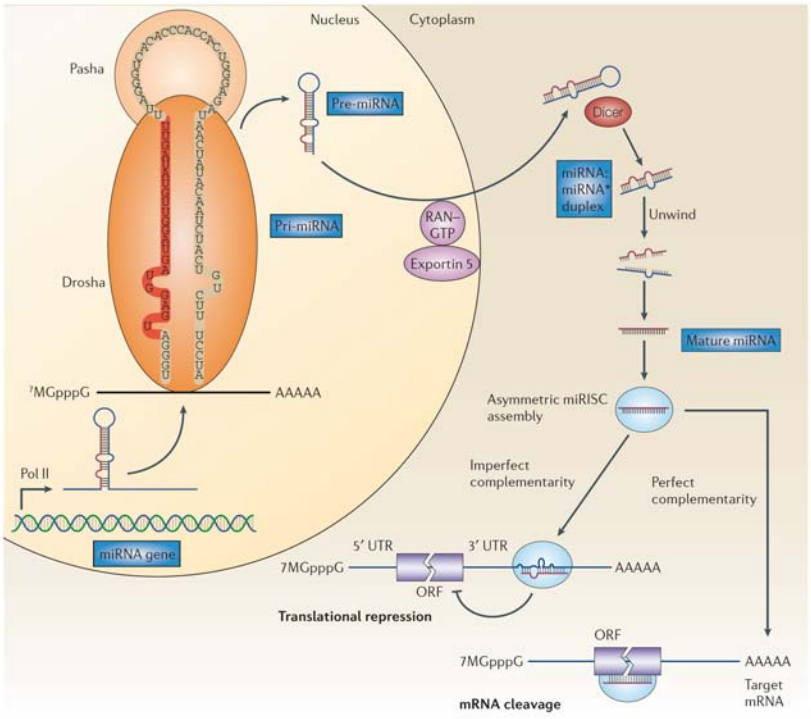


Fig.1.4.2a Biogenesis of microRNAs (figure from Slack F J., Nat.Rev.Cancer.,2006) This figure indicates the process of microRNA (miRNA) biogenesis in a cell. The blue boxes label the microRNA at different stages of biogenesis. In the nucleus, the miRNA gene is transcribed into a primary miRNA which is then acted upon by Drosha to form 70-80 nucleotide long precursor miRNA (Pre-miRNA). Pre-miRNA when exported out to cytoplasm, dicer acts on it to form a mature miRNA. This biogenesis process is followed by loading of the guide strand of the mature miRNA onto RISC for binding to the 3'UTR of a target mRNA.

generated as polycistronic primary transcripts. It is demonstrated by *in-vivo* and *in-vitro* studies that miRNA biogenesis takes place in two sequential steps: i) formation of 70-80nt long precursor miRNA (pre-miRNA) from primary miRNA (pri-miRNA) and ii) processing of pre-miRNA into mature miRNA (Kim, 2005; Lee et al., 2002). Mature miRNA sequences are contained within regions of pri-miRNAs that fold back on themselves to form approximately 60-80nt stem-loop structure as shown in the **Fig.1.4.2a.**

In the first step, the primary miRNA is acted upon by a microprocessor unit that includes Drosha, a nuclease of the RNAse III family and its binding partner DGCR8 as core components, in the nucleus to form 70-80nt long pre-miRNA (Denli et al., 2004; Gregory et al., 2004). The short stem of ~22 base pairs and a 2 nucleotide 3'overhang of pre-miRNA are recognized by the

nuclear export factor exportin5 (Exp5) (Bohnsack et al., 2004; Lund et al., 2004) to promote the correct and efficient processing of precursors in the generation of mature miRNAs. Exp5 may facilitate miRNA biogenesis by monitoring the integrity of pre-miRNAs and by promoting efficient release of pre-miRNAs from Drosha in the nucleus, where the level of RanGTP is high. The exported pre-miRNA undergoes further processing by one more RNA endonuclease III, Dicer, which removes the terminal loop, yielding a double-stranded 18-24nt RNA duplex. This duplex is further loaded onto a multicomponent protein complex known as the RNA-induced silencing complex (RISC). One of the strands of the duplex remains attached to the RISC while the other one gets destroyed. Selection of the RISC attached strand (referred to as guide strand) depends upon the base pairing at 5'end. The strand with less stable base pairing at 5'end becomes the mature miRNA (Khvorova et al., 2003; Schwarz et al., 2003).The fully processed miRNA duplexes are functionally equivalent to externally introduced siRNA.

1.4.2.3 Mechanism of miRNA regulation:

MiRNA guided strands, once loaded onto RISC, achieve gene silencing by either mRNA cleavage or translational repression of the target gene. The choice of mechanism depends upon the degree of complementarity between a miRNA and its target (Hutvagner and Zamore, 2002; Zeng et al., 2003). Unlike siRNAs, miRNAs in vertebrates usually recognize one or more imperfectly complementary binding sites in the target mRNA 3'UTR. The partial complementarity prevents the cleavage by RISC and results in translational repression of the mRNA. The core protein components of RISC that interact with miRNAs are the Argonaute (Ago) proteins (Carmell et al., 2002). Ago2 out of 4 members of Argonaute family (Ago1-4) is capable of directing the miRNA mediated mRNA cleavage (Liu et al., 2004; Meister et al., 2004). The PIWI domain of the Ago2 structurally resembles the RNase H and provides the endonuclease activity to RISC. Recently people have reported the probable mechanisms of inhibition of mRNA translation by miRNAs. It is proposed that the inhibition of mRNA takes place at the translation initiation step (Pillai et al., 2005). They demonstrated that endogenous let-7 microribonucleoproteins (miRNPs) or the tethering of Ago proteins to reporter mRNAs in human cells inhibit translation initiation. M^7G-cap-independent translation is not subject to repression, suggesting that miRNPs interfere with recognition of the cap. The other probable mechanism is inhibition at the translation elongation step, wherein translational repression by miRNAs is

primarily due to ribosome drop off during elongation of translation (Petersen et al., 2006). The data demonstrated that gene silencing occurs before completion of the nascent polypeptide chain. In addition, silencing by short RNAs causes a decrease in translational readthrough at a stop codon, and ribosomes on repressed mRNAs dissociate more rapidly after a block of initiation of translation than those on normal mRNAs. Thus, miRNAs regulate the expression of their target genes post-transcriptionally by translational repression.

1.4.2.4 MiRNAs in cancer:

Till date, hundreds of miRNAs have been identified in human genome. Many of these miRNAs have been implicated in different diseases including cancers. Since miRNAs can simulatenously regulate the expression of a number of target genes, deregulation of a single miRNA may have a strong impact on important regulatory pathways in cancer. Kent and Mendell reviewed different reports which have shown the dysregulation of miRNAs in diverse cancers. (Kent and Mendell, 2006).Ongoing research in the field of miRNAs demonstrate the function of miRNAs as tumor supressors or oncogenes in different cancers. Interestingly, it was recently shown that 50% of annotated human miRNAs are located at fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions (Calin et al., 2004b) of human chromosomes which indicates a direct association of miRNAs with cancer progression.

MiRNAs as oncogenes:

As discussed above, miRNAs negatively regulate the expression of their target genes, miRNAs acting as oncogenes downregulate the expression of their tumor suppressor targets leading to oncogenesis. Sonoki and colleagues reported a patient with precursor-B-cell acute lympho-blastic leukemia who carried an insertion of pre-miRNA into the IgH locus (Sonoki et al., 2005) and supported the role of miRNAs as oncogenic microRNAs termed oncomirs in cancer. *BIC*/miR-155 was the first miRNA transcript shown to possess oncogenic or tumor promoting activity (Eis et al., 2005). Overexpression of BIC/miR-155 has consistently been observed in tumors such as breast, lung, colon and thyroid cancers including B-cell lymphoma (Eis et al., 2005; He et al., 2005a; Volinia et al., 2006). It was shown that miR-155 can act as an oncogene in lymphoma using an animal model (Costinean et al., 2006). E(μ)-mmu-miR155

transgenic mice were shown to exhibit pre-B cell proliferation followed by lymphomagenesis. miR-21 is also shown to act as antiapoptotic gene in numerous cancers including glioblastoma. Markedly elevated miR-21 levels have been observed in human glioblastoma tumor tissues, primary tumor cultures, and in glioblastoma cell lines compared with nonneoplastic fetal and adult brain tissues and primary cultured neurons and astrocytes (Chan et al., 2005). Recently, it has been reported that miR-21 regulates multiple genes associated with glioma cell apoptosis, migration, and invasiveness, including the *RECK* and *TIMP3* genes, which are suppressors of malignancy and inhibitors of matrix metalloproteinases (MMPs) (Gabriely et al., 2008). Like miR-155 and miR-21, there are many more miRNAs that are proven to be oncomirs.

In addition to miRNAs themselves, the components associated with them such as Ago proteins also act as an antiapoptotic genes. For example, expression of Ago1 in developing lung and kidney and its increased expression in renal tumors that lack the Wilms-tumour suppressor gene, *WT1* indicate its role in the differentiation of these tissues during embryogenesis (Carmell et al., 2002). An additional Ago gene, HIWI is mapped to the locus 12q24.33 which is a locus that is associated with testicular germ-cell cancers (Qiao et al., 2002).

MiRNAs as tumor-suppressors:

Unlike oncomirs, miRNAs acting as tumor suppressors downregulate various oncogenes and inhibit the process of tumor progression. miR-15a and miR-16-1 are some of the first miRNAs shown to be causatively related to cancer development. Human homologs of the *C.elegans let-7* miRNA are also been reported to have tumor suppressor activity. Mendell reviewed that humans possess twelve let-7 homologs organized in eight distinct clusters. Further it was reported that at least four of these clusters are located in genomic regions known to be deleted in cancer (Calin et al., 2004b). It has been demonstrated that let-7 expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors, providing a possible mechanism for let-7 in cancer (Johnson et al., 2005). The 3'UTR of the RAS gene bears multiple let-7 complementary sites allowing let-7 to regulate *RAS* expression. Further, miRNAs of the let-7 family are also proven to be involved in gastric cancer. They are proposed to target the HMGA2 gene and reduce its expression leading to inhibition of tumor invasiveness (Motoyama et al., 2008). Decreased expression of miR-143 and miR-145 has been demonstrated to be a frequent feature of colorectal tumors (Michael et al., 2003). These miRNAs are known to

be contained within a genomic interval frequently deleted in myelodysplastic syndrome (Calin et al., 2004a). It was later demonstrated that these two miRNAs are suppressive to cell growth in cervical cancer. Thus, further insights into the role of microRNAs and the identification of microRNA::target interactions will be important for designing therapeutic drugs for diseases including cancers especially in cases which present deregulation of microRNA mediated control of gene expression as a pathological event.

1.4.2.5 MiRNAs in cell cycle regulation:

In addition to oncogenesis, miRNAs play an important role in cell cycle regulation though the mechanism is still not uncovered. They may participate in cell apoptosis, survival and differentiation. MiRNAs involved in cell cycle regulation are a part of the cell cycle regulatory pathways and machinery. MiRNAs have been shown to be intricately linked to the regulation of cell cycle regulatory genes and the disruption of this fine-tuned apparatus has been linked to cancer. Recently, there have been many reports indicating the oncogenic potential of miR-221 and miR-222 by increased cell proliferation in cancers such as thyroid, prostate cancer and glioblastoma (Galardi et al., 2007; Gillies and Lorimer, 2007; le Sage et al., 2007; Visone et al., 2007). These miRNAs target the cell cycle suppressive p27 gene thereby increasing the cell proliferation and tumor progression. Ricardo and colleagues extended these studies by showing that these miRNAs are up-regulated upon exit from quiescence and they target p57 in addition to p27. (Medina et al., 2008). Another miRNA, miR-34a has also been involved in cell cycle regulation through p53 pathway. It is the direct target of p53, and induced as a transcriptional activation by p53. Expression of miR-34a causes dramatic reprogramming of gene expression and promotes apoptosis. Much like the known set of p53-regulated genes, miR-34a-responsive genes are highly enriched for those that regulate cell cycle progression, apoptosis, DNA repair, and angiogenesis (Chang et al., 2007; Raver-Shapira et al., 2007).Moreover, deletion of the chromosomal region 1p36 where the miRNA transcript is located is a common feature of numerous human cancers including neuroblastoma and pancreatic cancer which makes it an attractive candidate tumor suppressor. Recent studies showed decreased expression of miR-34a in colon cancer patients (Tazawa et al., 2007) and also evidenced that miR-34a is a potent suppressor of proliferation through modulation of E2F signaling pathway. Thus, miR-34a

participates and fine tunes the program of genes involved in p53 mediated cell apoptosis. There are many such miRNAs that participate in cell proliferation, apoptosis and senescence.

1.4.2.6 MiR-15a and miR-16-1:

MiR-15a and miR-16-1 are some of the first miRNAs causally linked to cancer development as mentioned above. These are clustered together on human chromosome 13q14, the locus that is deleted in over half of B-cell chronic lymphocytic leukemia (CLL) cases and there has been significant effort to identify the putative tumor-suppressor genes located within this interval. Detailed deletion and expression analysis showed that miR-15a and miR-16-1 are located within a 30-kb region (13q14) which is recurrently lost in CLL, and that both genes are deleted or down-regulated in 68% of CLL cases (Calin et al., 2002).The germ-line mutation near miR-16-1 present in CLL cases is associated with reduced expression of these miRNAs though underlying mechanism of this effect is unclear. The role of these two miRNAs in tumorigenesis is revealed as they directly target the antiapoptotic gene, BCL2. This suggests that loss of function of miR-16-1 and miR-15a promotes increased expression of BCL2 leading to abnormal survival of CLL cells. Further reports have shown the frequent 13q14 deletions in other malignancies including mantle cell lymphoma, multiple myeloma and prostate cancer (Dong et al., 2001). All these observations suggest the probable tumor suppressive function of miR-15a and miR-16-1 in numerous cancers.

Apart from BCL2, some transcripts containing AREs are shown to be targeted by miR-15a and miR-16-1. These miRNAs seem to recognize and rapidly degrade those transcripts. AREs in the 3'UTR of these unstable mRNAs dictate their decay. Dicer1, Ago1 and Ago2, the components of miRNA processing and function are reported to be required for the degradation of mRNA containing AU-rich sequences of tumor necrosis factor-alpha. It is further observed that miR16, a human miRNA containing an UAAAUAUU sequence that is complementary to ARE sequences, is required for ARE-RNA turnover (Jing et al., 2005). The role of miR16 in ARE-RNA decay is sequence-specific and requires the ARE binding protein tristetraprolin (TTP). miRNA targeting of ARE, therefore, appears to be an essential step in tumorigenesis thus loss of ARE-mediated decay would be expected to increase the expression of some proto-oncogenes such as c-Fos, c-Myc and cyclin D1 which have AREs (Chen and Shyu, 1995). Moreover, miR-16 induces cell cycle arrest at G1 phase by regulating multiple downstream effectors

simultaneously (Liu et al., 2008). Recently, it was demonstrated that miR-16 downregulates cyclin D1, cyclin D3, cyclin E1 and CDK6 which are involved in G1 to S transition in cell cycle, leading to inhibition of cell proliferation. MiR-16 has also been shown to promote apoptosis in cell dependent manner.

1.4.2.7 MiR-17-92 cluster miRNAs:

A well studied miRNA cluster, closely associated with different cancers is the miR-17-92 cluster. This cluster of six miRNAs, miR-17-5p, miR-20a, miR-19a, miR-19b, miR-18a and miR-92 is located at 13q31-32. The primary transcript encoding miR-17 cluster miRNAs occupies around 1kb of genomic locus forming *c13orf25*.

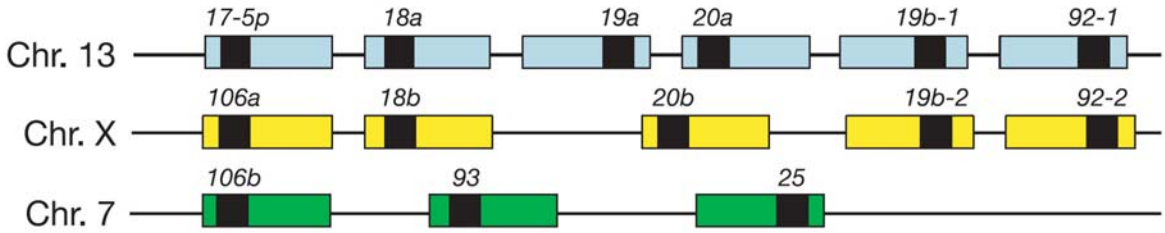


Fig.1.4.2b Schematic representation of the miR-17, miR-106a and miR-106b clusters. (figure from O'Donnell KA., Nature,2005) These are the three paralog clusters of miRNAs found in mammals on chromosome 13, X chromosome and on chromosome 7. The position of a mature miRNA is indicated by a dark box in a colored box with the name of a mature miRNA on top of the box.

This genomic locus is known to be amplified in multiple haematologic malignancies such as B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and primary cutaneous lymphoma (Ota et al., 2004; Rinaldi et al., 2007). MiRNAs of mir-17-92 family are overexpressed in lymphoma cell lines harbouring the amplification of this genomic region (He et al., 2005b). The significance of this cluster of miRNAs in cancer is further strengthened by the observation that the transcription of the *mir-17* cluster is directly activated by c-myc (O'Donnell et al., 2005). C-myc binds directly to the transcript of the mir-17 cluster and activates it. Further, miR-17-5p and miR-20a bind to the 3'UTR of the E2F1 transcription factor and decrease its protein expression. Thus, c-myc simultaneously activates E2F1 transcription and limits its translation, allowing a tightly controlled proliferative signal. Independently, Tagawa et al.

reported that the deregulation of miR-17 cluster and c-myc synergistically contribute to aggressive cancer development (Tagawa et al., 2007). They showed that nude mice injected with rat-1 cells transfected with miR-17 cluster along with c-myc present more accelerated tumor growth than those injected with myc transfectant cells. In another study, expression of miR-17 was demonstrated to increase proliferation in lung cancer cell line (Hayashita et al., 2005). Recently, a novel cyclin D1/miR-17/20a feedback loop has been demonstrated through which cyclin D1 induces miR-17-5p and miR-20a. Cyclin D1 binds to the miR-17 cluster promoter regulatory region and induces its expression, in turn miR-17-5p and miR-20a bind to the complementary site in the 3'UTR of cyclin D1 leading to inhibition of proliferation in breast cancer cells (Yu et al., 2008).

1.5 Aim of the study:

The dysregulation of cell cycle control is one of the fundamental aspects of cancers. Unlike normal cells, tumor cells proliferate as they are no longer subject to proliferation-inhibitory influences. Several components of the cell cycle regulatory machinery are targets for aberrant alterations leading to malignancy. Cyclin D1 is one such candidate which has been shown to be involved in tumorigenesis. The role of this gene in cancer can be gauged from its frequent dysregulation due to mutations, activation or overexpression in several human cancers such as breast cancer, small cell lung cancer, neuroblastoma and mantle cell lymphoma (MCL). In MCL, the characteristic t(11;14) translocation juxtaposes the cyclin D1 gene downstream of the immunoglobulin heavy chain enhancer that leads to overexpression of cyclin D1. Even though mutations that alter the coding sequence of this gene have rarely been reported in cancer, there are several reports that point to somatic mutations in the 3'UTR of the cyclin D1 mRNA although the contribution of this 3'UTR loss to the malignant process is underappreciated and unexplored. The pathological role of 3'UTR mutations in various diseases is becoming increasingly apparent making this an attractive regulatory region for systematic exploration in various diseases. The purpose of the study is the assessment of the regulatory potential of the cyclin D1 3'UTR, the characterization of various elements in the 3'UTR and their contribution to cyclin D1 expression, and the elucidation of the effect of the loss of this region on cyclin D1 expression and function.

Materials

2.1 Mammalian cell lines:

HeLa:	Human cervical cancer cell line
A549:	Human lung carcinoma cell line
MCF-7:	Human breast adenocarcinoma cell line
MDA-MB-453:	Human breast carcinoma cell line-(procured from DSMZ, Europe)
Phoenix Eco:	293 derived viral packaging cell line (kind gift from Dr. Slany, Erlangen)
NIH3T3:	Mouse fibroblast cell line
IEC-18:	Rat intestinal epithelial cell line-(procured from DSMZ, Europe)

2.2 Media and reagents:

2.2.1 Cell and tissue culture:

Media: Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/l glucose, l-glutamine, without sodium pyruvate and 3.7 g/l $NaHCO_3$ and Roswell-Park-Memorial-Institute 1640 (RPMI1640) with L-Glutamine and 2.0g/l $NaHCO_3$ Medium (PAN Biotech GmbH, Aidenbach, Germany)

Fetal Bovine Serum (FBS): 0.1 μm sterile filtered (PAN Biotech GmbH, Aidenbach, Germany)

Trypsin/EDTA: 0.05/0.02% lg in PBS without $Ca^{+2}Mg^{+2}$ (PAN Biotech GmbH, Aidenbach,Germany)

Dulbecco's phosphate buffered saline (DPBS): With calcium and magnesium (PAN Biotech GmbH, Aidenbach, Germany)

Penicillin/Streptomycin: 10,000 U Penicillin/ ml, 10 mg Streptomycin/ ml (PAN Biotech GmbH, Aidenbach, Germany)

Ciprofloxacin: Ciprofloxacin 400 solution, (Bayer AG, Leverkusen, Germany)

Polyfect transfection reagent: Reagent for DNA transfection in cell lines (Qiagen GmbH, Hilden, Germany)

Lipofectamine 2000 reagent: Reagent for miRNA and siRNA transfections in cell lines (Invitrogen, Carlsbad, CA)

Pre-miR miRNA Precursor Molecules (Pre-miRs): (Ambion, Cambridgeshire, UK)

Calcium chloride solution for transfection: 2.5 M $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) solution in water.

Hepes Buffered Saline: (HBS) (Invitrogen, Carlsbad, CA)

Protamine sulfate: (Salamine) from Salmon, cell culture tested (Sigma-Aldrich, St. Louis, MO) 5 mg/ml stock solution

Propidium iodide solution (PI): 50µg per ml solution for cell cycle analysis (Sigma-Aldrich, Steinheim)

RNase A solution: 50mg per ml solution for cell cycle analysis (Invitrogen Life Technologies, Carlsbad, CA)

Ethanol: For fixing of cells in cell cycle analysis (Merck KGaA, Darmstadt, Germany)

Fluorescence Activated Cell Sorting (FACS) buffer: 2%FBS and 2µg/ml PI in PBS.

Trypan blue stain: Trypan blue stain 0.4% for cell proliferation assay to check the viability of cells (Invitrogen, Carlsbad, CA)

Cell scrapers: 25 cm sterile cell scrapers (Sarstedt, Newton, NC)

Cell culture pipettes (2, 5, 10 and 25 ml): Sterile disposable pipettes (Corning Inc., Corning, NY)

Filtration units: Millex syringe driven filter units 0.22 µm and 0.45 µm filters (Millipore,Billerica, MA)

Cell culture plates and dishes: Sterile 6 well plates (Sarstedt, Numbrecht, Germany) 100 mm x 20 mm dishes for adherent cells (Corning Inc., Corning, NY, USA), and Tissue culture flasks (T-75 PE vented cap) for adherent cells (Sarstedt, Numbrecht, Germany)

2.2.2 Molecular biology:

Agarose: Electrophoresis grade (Invitrogen life technologies, Carlsbad, Germany))

Gel elution of DNA and PCR or DNA cleanup: Gel elution and PCR purification kit for DNA elution from gels and clean up of PCRs (Qiagen GmbH, Hilden, Germany)

Small-scale plasmid preparation: GFX miniprep kit for isolation of plasmid DNA from bacteria (Amersham Biosciences GmbH, Freiburg, Germany)

Endo free Plasmid Maxi Kit: Maxi prep kit for isolation of DNA from bacteria (Qiagen GmbH, Hilden, Germany)

RNeasy Mini Kit (250): RNA isolation from cells **(**Qiagen GmbH, Hilden, Germany)

RNase-Free DNase Set (50): For removal of DNA during RNA isolation (Qiagen GmbH, Hilden, Germany)

Mutagenesis kits: QuickChange II XL Site-Directed Mutagenesis kit for single site mutagenesis and QuickChange multi Site-Directed Mutagenesis kit (Stratagene, La Jolla CA, USA)

Molecular weight markers: Nucleic acid size standards, 1 kb ladder, 1 kb plus ladder and 100 bp ladder (Invitrogen, Carlsbad, CA)

Enzymes: *XbaI, ScaI, PstI, EcoRI, BamH1* and *HindIII*, all from New England Biolabs (NEB, Beverly, MA) Calf intestine phosphatase (CIP) and T4 DNA Ligase from Promega Corporation, Madison WI, USA

DNA polymerase: Ampli Taq Gold (250 units) 5 U/µl (Applied Biosystems, Foster City, CA USA) and Pfu Turbo (Stratagene, La Jolla CA, USA)

DMSO: Reagent for freezing cell lines (Sigma-Aldrich-Chemie GmbH, Steinheim, Germany)

dNTP mix: 100 mM each of dATP, dTTP, dCTP and dGTP (Invitrogen, Carlsbad, CA)

MgCl2 solution: 25mM MgCl2 solution (Applied Biosystems, Foster City, CA USA)

PCR buffer: 10X PCR Gold Buffer (Applied Biosystems, Foster City, CA USA)

RT-PCR kit: Gene Amp Gold RNA PCR Core kit (PE Biosystems, Foster City, USA)

ABsolute SYBR Green ROX Mix: For RQ-PCR reaction for *cyclin D1* mRNA expression analysis (Thermo Fisher Scientific Germany Co & Ltd., Bonn, Germany)

Reporter assay kit: Dual-Luciferase Reporter Assay System (Promega Corporation, Madison WI, USA)

2.2.2.1 Plasmids:

MSCV-IRES-GFP (pMIG): A modified form of the MSCV vector, it contains a bicistronic GFP expression cassette with an internal ribosomal entry site.

pEYFP-N1: Mammalian expression vector used for tagging genes with fluorescent reporter (Invitrogen, Carlsbad, CA)

pGL3-Control: Firefly luciferase expression vector for dual luciferase reporter assay (Promega Corporation, Madison WI, USA)

pGEM-T: Cloning vector (Promega Corporation, Madison WI, USA)

pcDNA3.1/V5-His-TOPO: Expression vector used for cloning the miR-17-92 cluster region (Invitrogen, Carlsbad, CA)

pRL-null: *Renilla* luciferase expression vector for dual luciferase reporter assay (Promega Corporation, Madison WI, USA)

2.2.2.2 Oligonucleotides:

All nucleotides were synthesized by Metabion AG, Martinsried, Germany.

Cloning primers:

CCND1 3'UTR-Fw	CGCTCTAGAGATACCAGAAGGGAAAGCTTC
CCND1 3'UTR-R(3191)	GGGGTTTTACCAGTTTTATTT
Cyd1_1kb_cDNA_Fw	GGCGAATTCCACACGGACTACAGGGGAGTT
Cyd1_1kb_cDNA_R2	GCGGGATCCCGGATGTCCACGTCCCGCAC
D1_miR_clus1_Fw	GGCTCTAGAACCTGTTTATGAGATGCTG
D1_miR_clus1_Rev	CGCTCTAGACCTACACCTATTGGACTGAA

D1_miR_clus2_Fw	CGGTCTAGACTTATTGCGCTGCTACCGTT
D1_miR_clus2_Rev	GCCTCTAGACATGGCTAAGTGAAGCATGAG
cyd1AURE-xhoI Fw	CGGCTCGAGGATGTTTCACACCGGAAGG
cyd1AURE-xhoI Rev	CCGCTCGAGCCTTCAACACTTCCTCCTAAAT
pcDNA6-int-Rev	GTTAGGGATAGGCTTACCTTCG

Mutagenesis primers:

Cy1-3utr-1insert_Fw	CTACAGATGATAGAGGATTTTATACCCCAATAAATCAACTCGTTTTTATAT
Cy1-3utr-1insert_Rev	ATATAAAAACGAGTTGATTTATTGGGGTATAAAATCCTCTATCATCTGTAG
Cy1-3utr-3delns_Fw	GTGCTACAGATGATAGAGGATTTTATACCCCAATAAACTCGTTTTTATATTAATG
Cy1-3utr-3delns_Rev	CATTAATATAAAAACGAGTTTATTGGGGTATAAAATCCTCTATCATCTGTAGCAC
AUREmut t1425c_t1427g	ACACTAAAATATATAATTTATAGTTAAGGCTAAAAAGTATACTGATTGCAGAGGATGTTCATAAGG
AUREmut t1459c_t1461g	GGATGTTCATAAGGCCAGTATGACTGATAAATGCAATCTCCCCTTGATT
AUREmut t1483c_t1485g	TTTATAAATGCAATCTCCCCTTGACTGAAACACACAGATACACACACACAC
AUREmut t1400c_t1402g	TTCACACCGGAAGGTTTTTAAACACTAAAATATATAACTGATAGTTAAGGCTAAAAAGTATAC
AUREmut t1548c_t1550g	ACAAACCTTCTGCCTTTGATGTTACAGACTGAATACAGTTTATTTTTAAAGATAGATCC
AUREmut t1815c_t1817g-Fw	CACATCTTGGCTATGTAATTCTTGTAATTTTTACTGAGGAAGTGTTGAAGGGA
AUREmut t1815c_t1817g-Rev	TCCCTTCAACACTTCCTCAGTAAAAATTACAAGAATTACATAGCCAAGATGTG
AUREmut t2669c_t2671g-Fw	TGTTTGTTATTGTTTTGTTAATTACACCATAATGCTAACTGAAAGAGACTCCAAATCTCAATG
AUREmut t2669c_t2671g-Rev	CATTGAGATTTGGAGTCTCTTTCAGTTAGCATTATGGTGTAATTAACAAAACAATAACAAACA

miR17seed del_Fw	CTAAAACCATTCCATTTCCAAGTTCAGTCCAATAGGTGT AGGAA
miR17seed del_Rev	TTCCTACACCTATTGGACTGAACTTGGAAATGGAATGGT TTTAG
miR15-16seed del-1_Fw	GCTCCATTTTCTTATTGCGTACCGTTGACTTCCAGGCA
miR15-16seed del-1_Rev	TGCCTGGAAGTCAACGGTACGCAATAAGAAAATGGAGC
miR15-16seed del-2_Fw	CTGTGTATCTCTTTCACATTGTTTGTATTGGAGGATCAGT TTTTTGTTTT
miR15-16seed del-2_Rev	AAAACAAAAAACTGATCCTCCAATACAAACAATGTGAA AGAGATACACAG

Sequencing primers:

cyD1 3'UTR-F2	CCCCCTTCCATCTCTGACTT
cyD1 3'UTR-R7	ACGTCAGCCTCCACACTCTT
luc-Cfor	AGAGAGATCCTCATAAAGGC
cyD1 3'UTR-R5	TCATCCTGGCAATGTGAGAA
cyD1 3'UTR-F7	CCTAAGTTCGGTTCCGATGA
cyD1 3'UTR-R8	CCAAAGCAGGCAGAACCTG
MIG Fw	TCCCTTTATCCAGCCCTCACTC

Real time RT-PCR primers:

CCND1-RT-Fw	AATGACCCCGCACGATTTC
CCND1-RT-Rev	TCAGGTTCAGGCCTTGCAC
TBP-RT-Fw	GCACAGGAGCCAAGAGTGAA
TBP-RT-Rev	TCACAGCTCCCCACCATGTT

Pre-miRs:

All Pre-miRs were synthesised by Ambion Ltd. Cambridgeshire, UK

Pre-miRs (microRNA mimics) sequences:

	Mature sequences corresponding to Pre-miRs
Pre miR-15a	UAGCAGCACAUAAUGGUUUGUG
Pre miR-16-1	UAGCAGCACGUAAAUAUUGGCG
Pre miR-95	UUCAACGGGUAUUUAUUGAGCA

2.3 Western blotting:

2.3.1 Reagents for western blotting:

Protein molecular weight markers: SeeBlue®Plus2 Prestained Standard (Invitrogen GmbH, Karlsruhe, Germany)

Bio-Rad protein assay Standard I: Lyophilized Bovine Plasma Gamma Globulin (Bio Rad Laboratories, Munich, Germany)

Dye reagent concentrate: Protein assay solution for protein estimation (Bio Rad Laboratories, Munich, Germany)

30% acrylamide mix: Rotiphorese Gel 30 (37.5:1) (Carl Roth GmbH, Karlsruhe, Germany)

TEMED: For SDS-polyacrylamide gel preparation (Sigma Aldrich Chemie GmbH, Germany)

2X SDS loading buffer: 125 mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto β-Ethanol and a pinch of Bromophenol blue

RIPA lysis buffer: 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 5mM EDTA and 50mM Tris pH 8.0

Milk powder: Blotting grade (Carl Roth GmbH, Karlsruhe, Germany)

Hydrogen peroxide: For ECL solution (Sigma Aldrich Chemie GmbH, Germany)

Standard Buffer solutions:

Membrane stripping-buffer	Beta-Mercaptoethanol 1M Tris-HCl – buffer pH6.6 SDS (20% w/v) Distilled water	360µl 2.5ml 4ml to make 40ml
10x SDS-PAGE buffer	Tris Glycin SDS Distilled water	150g 720g 50g to make 5l
Western blot transfer-buffer	Tris Glycin Methanol Distilled water	15.15g 75g 1l to make 5l
1.5M Tris-HCl (pH8.8 or pH6.8)	Tris Distilled water (pH adjusted with HCl)	908g to make 5l
10x TBS pH8.0	Tris Sodium chloride Distilled water (pH adjusted with HCl)	12.11g 87.66g to make 1l

Antibodies:

Name	**Company**	**Dilutions used**
Rabbit polyclonal anti-GFP	Invitrogen, Carlsbad, CA	1/ 5000
Mouse anti-human Cyclin D1	BD Biosciences, Pharmingen, NJ	1/ 500
Rabbit anti-Actin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	1/ 5000
Bovine anti-rabbit IgG-HRP	Santa Cruz Biotech. Inc., CA	1/ 5000
Bovine anti-mouse IgG-HRP	Santa Cruz Biotech. Inc., CA	1/ 5000

2.4 Instruments:

PCR and RT-PCR: GeneAmp PCR System 9700 for cloning PCR and RT-PCR (Applied biosystems, Foster City, USA)

Flow Cytometry: BD FACS calibur system (BD Biosciences, Palo Alto, CA)

Fluorescence Activated Cell Sorting: BD FACSVantage SE System (BD Biosciences, Palo Alto, CA)

Fluorescence microscope: Axiovert 200M (Carl Zeiss MicroImaging GmbH, Göttingen, Germany)

Microscope: Leitz Diavert Inverted Microscope (Opto Sonderbedarf GmbH, Munich, Germany)

Protein Electrophoresis: Power Pac 300 and power pac 1000 (Bio Rad Laboratories, Munich, Germany)

Dual Luciferase Assay Reporter measurements: Orion II microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany)

Blot development: Phospho imager CAWOMAT 2000 IR (CAWO Photochemisces Werk GmbH, Schrobenhausen, Germany)

2.5 Software:

The analysis of the cyclin D1 3'UTR for putative microRNA binding sites was performed using the TargetScan and PicTar microRNA target prediction software tracks loaded on the University of California Santa Cruz Genome Browser May 2004 (hg17) assembly (URL: http://genome.ucsc.edu). The analysis for the AURE sequences was performed by manually scanning AUUUA pentamers in the 3'UTR sequence of the human cyclin D1 gene downloaded from the University of California Santa Cruz Genome Browser May 2004 (hg17) assembly as well. The CellQuest software (BD biosciences, Paulo Alto CA, USA) was used for the calculation of mean fluorescent intensities (MFI) of YFP from different CCND1-YFP constructs transiently transfected into HeLa cells. The ModFit LT program (Verity Software House,

Topsham, ME, USA) was used for the cell cycle analysis of IEC-18 cells transduced with cyclin D1. Primers were designed using the Primer3 program, Whitehead Institute, Massachusetts Institute for Technology, MA (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Methods

3.1 Cloning of constructs:

Cyclin D1 (CCND1) 3'UTR:

The 3.19 kb CCND1 3'UTR was amplified from the genomic DNA of peripheral blood cells isolated from a healthy consenting individual using the primers CCND1 3'UTR-Fw and CCND1 3'UTR-R(3191), by Ampli Taq Gold DNA polymerase enzyme, using the following PCR program:

Temperature (^{o}C)	Time	Cycles
95	10 minutes	1
95	45 seconds	
56	45 seconds	8
72	3 minutes	
95	45 seconds	
58	45 seconds	8
72	3 minutes	
72	10 minutes	1
4	hold	

The amplicon was then ligated to the pGEM-T vector using T4 DNA ligase and incubated overnight at 4°C. After screening colonies transformed with the ligation mixture, the appropriate clone (pGEM-T-UTR) was identified by digestion of the minipreps by the restriction enzymes *Pst I* and *XbaI*. This clone was completely sequenced by cyD1 3'UTR-F7, cyD1 3'UTR-R5 and cyD1 3'UTR-R7 sequencing primers and subsequently sub-cloned as an *XbaI* digested fragment into the dephosphorylated *XbaI* site of the pGL3-Control. Competent *E.coli* (DH5α) were used for transformation of the ligation mixture and colonies were identified by colony PCR using the primers cyD1 3'UTR-F7 and cyD1 3'UTR-R8. The PCR program used was as follows:

Temperature (^{o}C)	Time	Cycles
94	2 minutes	1
94	30 seconds	25
55	30 seconds	
72	45 seconds	
72	5 minutes	1
4	hold	

Plasmid DNA was prepared from colonies showing amplification with the aforementioned primers using the small scale plasmid isolation kit. All the mini preps were then digested with *Hind III* to identify clones with the CCND1 3'UTR in the correct 5'to 3' orientation with respect to the luciferase gene. The correct clone harbouring the full length 3'UTR (Flu) was sequenced completely using luc-Cfor and cyD1 3'UTR-F7 sequencing primers.

CCND1 3'UTR fragments:

The region of CCND1 3'UTR including a highly conserved miR-17-92 cluster binding site (reg-17-92) and a region complementary to the two miR-15/16 binding sites (reg-15/16) were amplified from the full length cyclin D1 3'UTR clone (Flu) using the primer pairs D1_miR_clus1_Fw, D1_miR_clus1_Rev and D1_miR_clus2_Fw, D1_miR_clus2_Rev respectively. The PCR program used was as follows:

Temperature (^{o}C)	Time	Cycles
94	2 minutes	1
94	45 seconds	30
55	45 seconds	
72	1 minute	
72	5 minutes	
4	hold	

Both the above mentioned fragments (reg-17-92 and reg-15/16) were then digested with *XbaI* and ligated separately to the cut and dephosphorylated *XbaI* site of the pGL3-Control vector downstream of the luciferase gene. An individual colony PCR of the transformed ligation

mixtures was performed using the above respective cloning primers for the regions. The PCR program used was the same as that for cloning albeit with 20 cycles. The appropriate clones of the regions were identified by sequencing with the primer luc-Cfor.

MiR-17 cluster and sensor plasmids:

The miR-17 cluster was obtained as a clone in the pcDNA3.1/V5-His-TOPO expression vector. The sensor plasmids of miR-17-5p and miR-20a were made by cloning the sequences perfect complementary to the miR-17-5p and miR-20a miRNAs respectively in two tandem repeats into the pGL3-Control (O'Donnell et al., 2005). These constructs (CMV-miR-17 cluster, miR-17-5p-sensor and miR-20a-sensor) were a kind gift from Prof. Joshua Mendell at the Johns Hopkins Institute.

MiR-15/16 expression plasmid:

The miR-15/16 miRNAs cloned into expression plasmid (pLenti6-BLOCK-iT-DEST) (Linsley et al., 2007) were obtained as a kind gift from the Merck & Co., Inc.

CCND1 CDS in peYFP-N1:

The 1.2 kb CCND1 without 3'UTR (5'UTR and coding sequence) was first cloned into the pcDNA6/V5-HisA plasmid (CCND1 full-pcDNA6). CCND1 CDS was amplified using the cloning primers Cyd1_1kb_cDNA_Fw and Cyd1_1kb_cDNA_R2 from the aforementioned construct, using the following PCR program:

Temperature (^{o}C)	Time	Cycles
94	2 minutes	1
94	45 seconds	28
55	45 seconds	
72	1 minute	
72	5 minutes	1
4	hold	

The *EcoRI*, *BamHI* digested CCND1 CDS amplicon was further cloned into the *EcoRI*, *BamHI* digested peYFP-N1 vector fusing the cyclin D1 CDS in frame with the YFP at the N terminal end (CCND1 CDS-YFP).

CCND1 full gene into peYFP-N1 (CCND1 full-YFP):

The CCND1 3'UTR was subcloned downstream of the YFP in the CCND1 CDS-peYFP-N1 construct in a downstream *XbaI* site. For this, an *XbaI* - *XbaI* digested 3'UTR was ligated to the *XbaI* digested and dephosphorylated CCND1 CDS-YFP. Minipreps were made from the colonies picked from the transformed ligation mixture. The appropriate clone (CCND1 full-YFP) was confirmed by sequencing with the primer cyD1 3'UTR-R8.

CCND1 full gene with ARE deletion into peYFP-N1 (ΔARE-YFP):

The ΔARE-YFP construct was cloned by ligating the two fragments of the CCND1 3'UTR, The fragment before the first ARE sequence (F-1) and the one after the 6th ARE sequence (F-2) in the 3'UTR.

Firstly, the F-1 and F-2 were amplified from the Flu and CCND1 full-pcDNA6 respectively. The primer pairs CCND1 3'UTR-Fw, cyd1AURE-xhoI Rev and cyd1AURE-xhoI Fw, pcDNA6-int-Rev were used for F-1 and F-2 respectively. The following PCR program was used for both the fragments:

Temperature (^{o}C)	Time	Cycles
94	2 minutes	1
94	45 seconds	30
55	45 seconds	
72	1:50 minutes	
72	5 minutes	1
4	hold	

Both the amplicons were digested with *XhoI* and ligated using pGEM-T system for 1 hour at room temperature. Further this ligation mixture was used as a template to amplify CCND1 3'UTR without ARE region using the primer set CCND1 3'UTR-Fw, pcDNA6-int-Rev with the following PCR program:

Temperature (^{o}C)	Time	Cycles
94	5 minutes	1
94	45 seconds	
55	45 seconds	32
72	3 minutes	
72	10 minutes	1
4	hold	

The amplicon was gel eluted and *XbaI* digested to clone into the CCND1 CDS-peYFP-N1 construct.

CCND1 full gene and CCND1 CDS into pMIG:

The CCND1 full gene was sub-cloned into the MIG vector from CCND1 full-pcDNA6 construct. The PmeI digested CCND1 full-pcDNA6 was cloned into the *HpaI* site of the pMIG. The ligation mix of pMIG and CCND1 full gene was transformed into the *DH5α* and minipreps were made. These were further digested with *XhoI* to check the orientation of the clones. The correct clone (CCND1 full-MIG) was reconfirmed by sequencing with MIG Fw primer. Further the CCND1 full-MIG construct was digested with *XhoI* to remove the *XhoI*- *XhoI* fragment of the 3'UTR and religated at 4°c overnight to get the CCND1CDS-MIG construct (CCND1 without 3'UTR in pMIG).

3.2 Mutagenesis (CCND1 mutants):

MCL patient mutations:

The deletion of 3 bp 'TCA' or insertion of a nucleotide 'A' at the positions 309-311 and 308 respectively in the CCND1 3'UTR are the mutations found in MCL patient samples (Wiestner et al., 2007). These two types of point mutations were made individually into CCND1 full-peYFP construct using the QuickChange II XL Site-Directed Mutagenesis kit as per the manufacturer's protocol. The primer sets Cy1-3utr-3delns_Fw, Cy1-3utr-3delns_Rev and Cy1-3utr-1insert_Fw, Cy1-3utr-1insert_Rev were used for the deletion of 3 bp 'TCA' and insertion of

one nucleotide 'A' into the 100 ng of aforementioned construct. The following PCR program (as per the instructions in the manual) was used

Segment	Cycles	Temperature (^{o}C)	Time
1	1	95	1 minute
2		95	50 seconds
	18	60	50 seconds
		68	1 minute/kb of plasmid length
3	1	68	7 minutes

The PCR product was digested with *Dpn I* restriction endonuclease (10U/µl) (provided in the kit) to digest the parental DNA template. The digestion is followed by the transformation into XL10-Gold ultracompetent cells (provided in the kit) as per the protocol in the manual. The colonies from the agar plate were then inoculated into liquid cultures to make the minipreps. These minipreps were sequenced with cyD1 3'UTR-F2 primer to identify the appropriate mutants (CCND1-3del-YFP and CCND1-ins-YFP).

ARE sequence mutations (Flu-7ΔARE):

The pentamer sequence AUUUA is a signature motif of the ARE elements. 7 repeats of AUUUA sequences were mutated by substituting first U by C and the third one by G in each pentamer using the QuickChange Multi Site-Directed and Single Site directed Mutagenesis Kits. The cyclin D1 full gene with 5 mutated AREs in pGL3-Control vector (Flu- 5ΔARE) was made in two steps. firstly the AUREs at positions 1425-27, 1459-61 and 1483-85 were mutated using 100 ng each of the 3 primers AUREmut t1425c_t1427g, AUREmut t1459c_t1461g and AUREmut t1483c_t1485g into 100 ng of Flu as per the manufactirer's instructions. The PCR program (given in the manual) was as follows:

Segment	Cycles	Temperature (^{o}C)	Time
1	1	95	1 minute
2		95	1 minute
	30	55	1 minute
		65	2 minutes/kb of plasmid length

The PCR product was digested with *Dpn I* restriction endonuclease (10U/µl) (provided in the kit) to digest the parental DNA template. The digestion is followed by the transformation into XL10-Gold ultracompetent cells (provided in the kit) as per the protocol in the manual. The colonies from the agar plate were then inoculated into liquid cultures to make the minipreps. The minipreps were sequenced with cyD1 3'UTR-F12 to identify the correct mutant (Flu-3ΔARE). This same protocol including the PCR program was used to mutate further 2 ARE pentamers at positions 1400-02 and 1548-50 in the Flu-3ΔARE. 100 ng of Flu-3 ΔARE was mutated using 100 ng each of the primers AUREmut t1400c_t1402g and AUREmut t1548c_t1550g. The appropriate mutant (Flu-5ΔARE) was identified by sequencing the minipreps with cyD1 3'UTR-R7. The 6^{th} and the 7^{th} repeats of the AUUUA pentamer at position 1815-17 and 2669-71bp respectively were mutated by QuickChange II XL Site-Directed Mutagenesis kit using the primer pairs: AUREmut t1815c_t1817g-Fw, AUREmut t1815c_t1817g-Rev and AUREmut t2669c_t2671g-Fw, AUREmut t2669c_t2671g-Rev as per the manufacturer's protocol and the mutant Flu-7ΔARE was generated.

Mutant of the reg-17-92 clone (reg-17-92-M):

For mutations that generate mismatches in microRNA binding sites, 4 nucleotides of the sequence bearing perfect complementarity to the 7-8 nucleotide 'seed' sequence of the miR-17/20a miRNAs was replaced by randomly chosen non-complementary nucleotides using the QuickChange II XL Site-Directed Mutagenesis Kit. The mutant reg-17-92-M was amplified from 100 ng of reg-17-92 construct using the primers miR17seed del_Fw and miR17seed del_Rev with the PCR program mentioned in the section 3.2 for ARE sequence mutation, as per the manufacturer's protocol. The PCR product was further digested with *Dpn I* and transformed into XL10-Gold ultracompetent cells. The correct clone was identified by sequencing the minipreps with luc-Cfor sequencing primer.

Mutant of the reg-15/16 clone (reg-15/16-M3):

The two binding sites of the miR-15/16 miRNAs in the reg-15/16 were mutated by replacing the 4 nucleotides in the seed sequence of the miR-15/16 miRNAs by randomly chosen non-comlementary nucleotides, sequentially using the QuickChange II XL Site-Directed Mutagenesis Kit. The first binding site was mutated using the primers miR15-16seed del-1_Fw

and miR15-16seed del-1_Rev using 100 ng of the reg-15/16 as the template. The protocol was the same as that used for the generation of reg-17-92-M mutant. The correct mutant (reg-15/16-M1) was confirmed by sequencing using luc-Cfor primer.

The reg-15/16-M1 was used as the template for mutating the second binding site. The primers miR15-16seed del-2_Fw and miR15-16seed del-2_Rev were used for the PCR and the protocol used to generate the mutant was same as the one mentioned for reg-15/16-M1 generation. The appropriate mutant (reg-15/16-M3) was identified by sequencing with luc-Cfor primer.

3.3 Preparation of stable cell lines:

3 x 10^6 Phoenix Eco cells were plated in a 10 cm dish and on the following day used for transient transfection. Medium was changed 4 hours prior to the transfection and 20 µg plasmid DNA each of the gene of interest was added to sterile water and a sterile solution of 100 µl 2.5M $CaCl_2$ was added drop wise to the water-DNA mixture. The volume of water added initially was calculated so as to make the total volume 1 ml. This was added slowly to a tube containing 1 ml sterile Hepes buffered saline solution (pH 7.2). After gentle mixing and incubating at room temperature for 3-4 minutes, this mixture was added drop wise to the medium covering the whole plate and without agitating the cells. The medium was changed the next day and supernatant was collected from the cells every 12 hours (thrice totally) and fresh medium added. This supernatant was filtered with a 0.45 mm Millipore filter and stored as VCM at –80°C for later use or used directly to transduce NIH3T3 fibroblasts and IEC-18 epithelial cells.

5x 10^4 NIH3T3 fibroblasts and IEC-18 cells were plated into 6 well plates one day prior to transduction. The next day, medium was withdrawn from these cells and 500 µl or 1 ml of fresh or frozen VCM was layered on top of the cells with the addition of a final concentration of 10 µg/ml protamine sulfate. Fresh medium was added after 4 hours and the transduction procedure was repeated every 12 hours for three-four times. The cells were expanded and two days were allowed for GFP expression. Green fluorescent cells were sorted using the fluorescence activated cell sorter (FACS) sorter and propagated. Using these protocols, *CCND1*

CDS, only *GFP* and *CCND1 full* NIH3T3 and IEC-18 stable cell lines were constructed and used for experiments.

3.4 Proliferation assay:

Proliferation assay was performed in stable CCND1-NIH3T3 and CCND1-IEC-18 cell lines to check the phenotypes of the cells with CCND1 with and without 3'UTR. The cells expressing CCND1-CDS, CCND1 full and empty vector individually were plated at a density of $1X10^5$ each in 6 well plates and this was counted as day 0. The cells were trypsinized and living cells were counted every alternate day (on day2, day4, day6 and day8) using Trypan blue. The cell suspension and trypan blue are mixed in 1:1 proportion and the number of viable cells per milliliter of the cell suspension (cells /ml) was calculated using the following formula:

Total no. of viable cells (10^4) = (No. of viable cells / 4) X dilution factor X10, 000

3.5 Cell cycle analysis:

The effect of overexpressed *CCND1* with or without 3'UTR on cell cycle was checked by cell cycle analysis in the stably transfected IEC-18 primary cell line. After sorting and propagating GFP positive cells expressing *CCND1* with or without 3'UTR or empty vector pMIG Cells were harvested for cell cycle analysis. Approximately 1 x 10^6 cells were used for the analysis. These cells were washed with PBS twice and spun down for 5 min. at 1000 rpm. The supernatant was removed and the cells were resuspended in 50 µl of fresh PBS. 1 ml of ice cold 80% ethanol was added dropwise with constant vortexing, mixed well followed by 30 min. incubation on ice. Cells were then pelleted by centrifugation for 5 min. at 1000 rpm. Cells were again washed twice with PBS to remove ethanol completely. Cell pellet was loosened by light vortex and 500 µl of 50 µg/ml PI solution was added with constant vortexing, followed by addition of 500 µl of 1mg / ml RNase A solution. Cells were then incubated in the dark for 30 min. at room temperature and analysed on FACS caliber. The analysis was done using the software ModFit LT.

3.6 Western blotting:

Sample preparation and cell lysis (total cell extract)

Western blotting was done to show the CCND1 protein expression in CCND1 full and CCND1 CDS-NIH3T3 and IEC-18 stable cell lines and the expression of the *CCND1CDS* and *CCND1full-YFP* constructs. This was also done to check the *CCND1* protein expression in transiently transfected MCF-7 and MDA-MB-453 cell lines. The cells were lysed using 100 µl RIPA buffer with fresh added protease inhibitors and detached using a cell culture scraper. The cells with RIPA buffer were transferred to an Eppendorf microcentrifuge tube and mixed by inversion and kept on ice for 30 minutes. After the homogenization, the sample was centrifuged at 14000 rpm for 30 minutes at 4^{o}c. After centrifugation, the supernatant was transferred to a new Eppendorf tube and either frozen at -80°C, or kept on ice for determination of protein concentration. As a control, Granta cells overexpressing CCND1 protein were used. Lysate was prepared using the method described above.

Determination of Protein Concentration:

The method used for measuring the protein concentration was the Bradford method. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. Within the linear range of the assay (1-21 µg/ml), the more protein present, the more Coomassie binds. The protein concentration of the sample was determined by comparison to values obtained for the measure of the known range of protein standards. The protein standard used was Bovine Plasma Gamma Globulin. Six different gamma globulin concentrations (1.4µg, 2.8µg, 7µg, 14µg and 21µg) were diluted in distilled water to a final volume of 800 µl. Two microliter of cell lysate was diluted in distilled water for the measure. 200 µl of Protein Assay solution (Dye reagent concentrate) was added to the tubes. The tubes were incubated at room temperature for 15 minutes and the content was further transferred to polystyrol cuvettes. A determination of the standard curve of the spectrophotometer

with distilled water and the protein standards was done using the specific program for protein in the spectrophotometer. The sample was measured following the standard curve determination.

SDS PAGE of Cell Extracts:

Total cell extract (TCE) proteins were separated on a denaturing gel consisting of 10% Tris-glycine gel and a 5% stacking gel. The concentration of the separation gel was chosen considering the size of CCND1 protein. The sample was homogenized and diluted 1:1 with 2x loading buffer and heated at 95^{o}C for 2 minutes. 40 µg protein was loaded in each lane and the SeeBlue®Plus2 Prestained Standard in one of the lanes. The electrophoresis was performed in the 1x SDS-PAGE buffer under 60 V for 30 minutes and 80 V for 1 hour and 30 minutes using Power pac 1000.

Protein Blotting:

During the electrophoresis, the PVDF membrane was wetted in mthanol for 30 seconds, rinsed with distilled water and equilibrated in western blot transfer buffer. The blotting papers (6 for each gel) were equilibrated in western blot transfer buffer as well. After the electrophoresis, the gel was taken from the cassette and placed on the stack of 3 bloting papers and a PVDF membrane. 3 more blotting papers were stacked on the gel and the blotting was done at 250mA for 2 hours and 30 minutes. The observation of the high molecular weight proteins of the pre-stained protein standard on the membrane was an indicator of successful transfer.

Protein detection on the blotting membrane with HRP-marked antibodies:

The antibody-detection of protein was performed following the instructions of the antibody's supplier. After the transfer, the membrane was blocked to prevent non-specific binding of antibodies to the membrane by incubating with 5% milk solution in TBS with 0.05% Tween-20 (TBST) for one hour at room temperature.The membrane was further incubated with the primary antibody at 1:5000 dilution in 5%Albumin Fraction solution in TBST overnight at 4^{o}c. The concentration used for the antibodies was adjusted according to the intensity and background. After incubation with the primary antibody, the membrane was washed three times with TBST (each wash for 5 minutes) at room temperature on a roller. The secondary antibody conjugated with Horse Radish Peroxidase (HRP) was diluted 1:5000 in 5% milk TBST solution

and put on the membrane for 1 hour incubation at room temperature. The membrane was washed again three times with TBST on roller. To detect the antibodies on the membrane an ECL detection solution (4 ml SA solution, 1.2µl hydrogen peroxide and 400µl SB solution) was used. After washing, the ECL detection solution was put on the membrane for 2 minutes. The membrane was dried, covered with a plastic film and put in a cassette for exposure of the film. The film was put on the membrane in a dark room and the exposure was done at variable exposing times between 15 seconds and 1 minute, depending on the visualization signal observed.

3.7 Reporter assays:

The dual luciferase reporter assay system was used to show the direct and specific interaction between CCND1 3'UTR and miRNAs targeting it. HeLa cells were plated at a density of $3x10^5$ cells per well in 6-well plates one day before transfection. pGL3-Control, Flu, reg-17-92, reg-17-92-M, miR-17-5p-sensor, miR-20a-sensor, and CMV-miR-17 cluster were transfected transiently using 12 µl of Polyfect transfection reagent as described by the manufacturer in independent wells. Firefly luciferase activity from the Flu, pGL3-Control, reg-17-92, reg-17-92-M and sensor plasmids and Renilla luciferase activity from the internal control plasmid pRL-null were determined 24 hour after the initiation of the transfection protocols using the Dual-Luciferase reporter Assay System. Firefly luciferase activities were normalised to the Renilla luciferase values of pRL-null. Results are given as means ± S.E.M. of three independent experiments. The following DNA concentrations of the reporter constructs and expression plasmids were used for Polyfect transfections: 0.03 µg of the internal control pRL-null, 1.5µg of pGL3-Control, Flu, reg-17-92, reg-17-92-M, miR-17-5p-sensor, miR-20a-sensor each and 0.5µg of CMV-miR-17 cluster was cotransfected with each of the above reporter constructs. In the transfections without cotransfection of CMV-miR-17 cluster, empty vector pcDNA6/V5-HisA was included to equilize the amount of DNA in transfection set.

The same reporter assays were performed with 1.5 µg of reg-15/16, reg-15/16-M3 each cotransfected with 50nM pre-miR-15a, pre-miR-16-1 and pre-miR-95 each. 3µl of Lipofectamine

2000 transfection reagent was used for the aforementioned tranfections. Firefly luciferase activities were normalised to the internal control Renilla luciferase values of pRL-null.

The dual luciferase reporter assays were performed also to study the effect of AREs on cyclin D1 regulation. 1.5µg of each of Flu-7ΔARE and Flu was cotransfected with the internal control 0.03µg of pRL-null using 12µl of polyfect and the aforementioned protocol of the dual reporter assay was used.

3.8 Transient transfections and fluorescence microscopy:

Twenty four hours before transfection, HeLa cells were plated at a density of $3x10^5$ cells per well in 6-well plates. The cells then transiently transfected with 2µg of each of CCND1 full-YFP, CCND1-CDS-YFP and ΔARE-YFP construct using 3µl of Lipofectamine 2000 as per the protocol described by the manufacturer. 18-20 hour post transfection the cells were observed under the fluorescence microscope (Axiovert 200M) (10X) for the fluorescence of the transfected cells. Similar transfection efficiencies could be obtained with the different constructs. The observed cells were trypsinized with 1 ml of Trypsin/EDTA per well and suspended in 1 ml of FACS buffer per sample for FACS analysis.

3.9 Flow cytometry:

Quantitative estimation of the difference between expression of CCND1 protein with and without 3'UTR was done by flow cytometric analysis of transiently transfected HeLa cell (mentioned in section 3.8). The cells in FACS buffer were analysed on the BD FACS Calibur System for YFP positivity using the YFP filter and histograms were plotted using the CellQuest software for the calculation of mean fluorescence intensity (MFI) of YFP. The mean fluorescent intensities of the constructs with different UTRs were compared. All experiments were performed in triplicates.

3.10 Estimation of ectopically expressed cyclin D1 protein:

Twenty four hours before transfection, HeLa cells were plated at a density of $2x10^6$ cells per well in 10 cm dish for adherent cells. The cells then transiently transfected with 15µg of each of CCND1 full-YFP, CCND1-CDS-YFP using 50µl of Lipofectamine 2000 as per the manufacturer's protocol. Similar transfection efficiencies were achieved. 24 hour post transfection the cells were harvested for CCND1 protein expression by western blotting described in section 3.6. Only YFP tagged cyclin D1 protein expression was analysed using polyclonal anti-GFP antibody.

3.11 Estimation of endogeneously expressed cyclin D1 protein:

Effect of miRNAs of miR-15/16 and miR-17-92 family on endogenous cyclin D1 was studied in MCF-7 and MDA-MB-453 breast cancer cell lines. These cell lines were transiently transfected with 15µg of each of miR-17-CMV and miR-15/16 constructs individually. $2x10^6$ of MCF-7 and $5x10^6$ of MDA-MB-453 cells were plated in 10cm dish for adherent cells one day prior to transfection. The next day the aforementioned constructs of miRNAs were transfected using 50µl of lipofectamine 2000 according to the manufacturer's protocol. Mock transfections of Lipoectamine 2000 without miRNA constructs were also performed as a control. The cells were harvested after 48 hours of transfection for mRNA and protein expression analysis. Western blots were performed as described in section 3.6 using monoclonal mouse anti-human cyclin D1 antibody.

3.12 RNA isolation and cDNA synthesis:

48 hour post transfection with miRNAs of miR-17-92 or miR-15/16 family, MCF-7 and MDA-MB-453 cells were harvested for RNA isolation. RNA isolation was performed using the RNeasy mini kit as per the manufacturer's protocol. DNA was removed from RNA using the RNase-Free DNase Set during RNA isolation as per the given protocol. This DNA free RNA sample was used for cDNA synthesis. A 20µl reaction contains 4 µl of 5X buffer, 4 µl of $MgCl_2$,

8 µl of deoxynucleoside triphosphate mix and 1 µl of random hexamer primer. To this, 1 µl of RNase inhibitor, 1 µl of Reverse transcriptase enzyme and 1 µl of DNA free RNA were added and RT-PCR was done using the following program:

Temperature (^{o}C)	Time (minutes)
25	10
42	30
99	5
4	0.5
4	90

3.13 Real-time RT-PCR:

cDNA prepared by reverse transcription was then used for *cyclin D1* mRNA expression analysis by Taqman^{TM} real time quantitative polymerase chain reaction (RQ-PCR) method. 2 µl of cDNA was added to the 25 µl reaction containing 12.5 µl of 2X Absolute SYBR Green Rox mix, 2 µl of each primer and 6.5 µl of water and PCR was done on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The CCND1-RT-Fw and CCND1-RT-Rev primers were used to measure the relative expression of CCND1 mRNA in MCF-7 and MDA-MB-453 cells. The CCND1 mRNA expression was normalized to TATA box binding protein (TBP) mRNA expression using the primer pair: TBP-RT-Fw and TBP-RT-Rev.

Results

4.1 Loss of 3'UTR leads to increased cyclin D1 expression:

In order to identify whether the loss of the cyclin D1 3'UTR could contribute to the enhanced cyclin D1 protein expression observed in human malignancies, we transiently transfected HeLa cells with expression plasmids expressing the full length cyclin D1 cDNA (CCND1 full-YFP) or the cyclin D1 transcript without the 3'UTR (CCND1 CDS-YFP) **(Fig.4.1a)**. These transcripts were tagged with YFP to enable tracking of transfected cells by fluorescence microscopy and for quantitative estimation of expression levels of the cyclin D1 protein by flow cytometry.

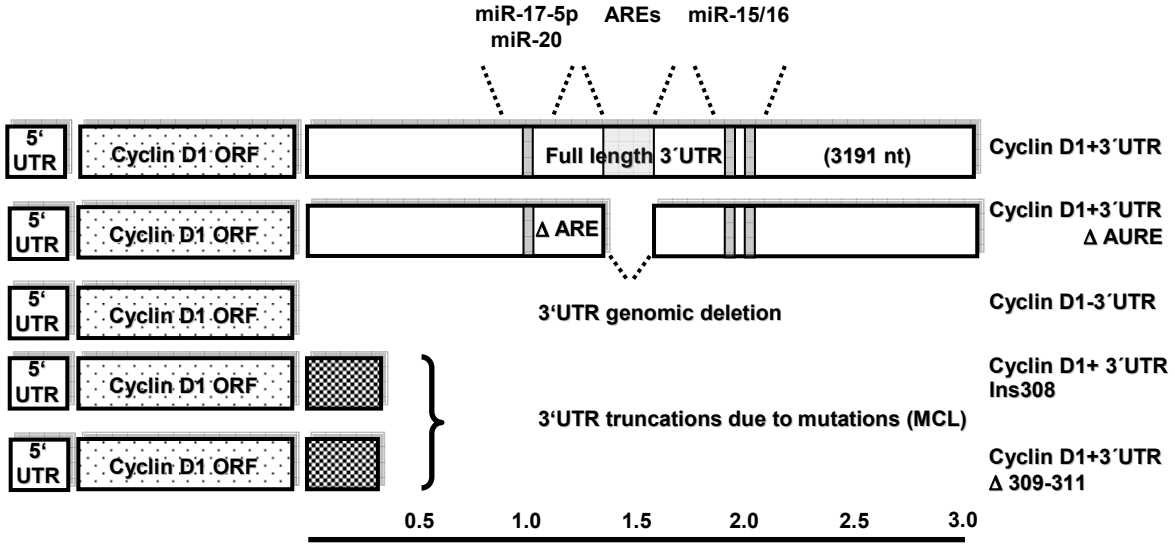
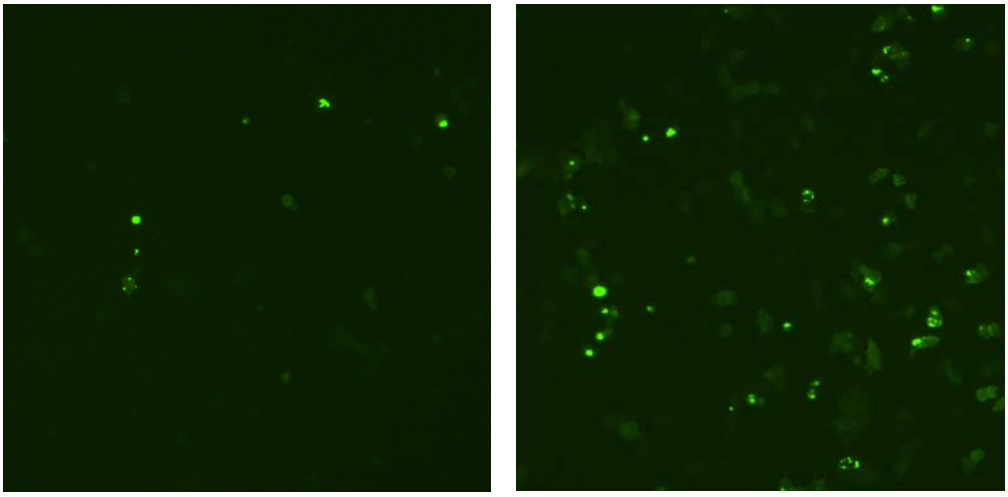


Fig.4.1a Different naturally occurring and patient specific forms of the *cyclin D1* mRNA: The *cyclin D1* transcript is 4.5 kb with the 3'UTR spanning 3.1 kb. An approximately 467 nt AU-rich region in the 3'UTR harbours several signature ARE elements. In MCL as well as in some other cancers, genomic deletions of the 3'UTR have been reported. In addition, point mutations that introduce premature polyadenylation, thereby truncating the UTR have been reported in MCL. Bioinformatic analysis of the long (3.1 kb) 3'UTR of cyclin D1 reveals potential microRNA binding sites for microRNAs. In contrast, the cancer associated isoforms of *cyclin D1* with the shorter 3'UTR do not harbour these sites suggesting that they are not subject to regulation by these microRNAs. (Note: scale drawn in the figure is an approximation).

Interestingly, protein expression was significantly higher upon deletion of the cyclin D1 3'UTR as assessed by fluorescence microscopy (**Fig.4.1b**) as well as quantitatively (2.13±0.057 fold) using flow cytometry (n=3, P=0.01) (**Fig.4.1d**). This shows that the cylinD1 3'UTR is important for the regulation of cyclin D1 expression and the loss of this region could contribute to elevated levels of this protein.



Cyclin D1+3´UTR **Cyclin D1-3´UTR**

Fig.4.1b Increase in Cyclin D1 protein expression upon deletion of the 3'UTR: Fluorescence microscopic pictures of Hela cells transfected with cyclin D1-YFP with or without the 3'UTR.

We also tested the levels of the cyclin D1 protein by Western blotting in these transiently transfected HeLa cells. Here also it could be seen that the levels of cyclin D1 protein were much higher in cells transfected with cyclin D1 without the 3'UTR confirming that the loss of this region can lead to cyclin D1 protein overexpression (**Fig.4.1c**).

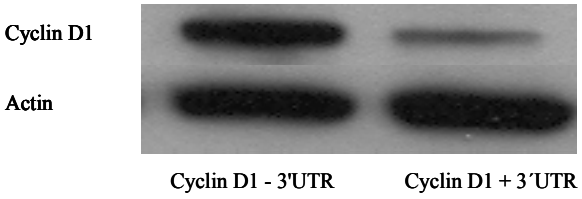


Fig.4.1c: Cyclin D1 protein expression is increased in cells expressing cyclin D1 without the 3'UTR as compared to those expressing full length cyclin D1: Western blots of HeLa cells transfected with cyclin D1-YFP with or without the 3'UTR.

Since the loss of the cyclin D1 3'UTR can lead to elevated levels of cyclin D1, genomic deletions of this region which have been reported in different tumors could result in the observed cyclin D1 overexpression. In some patients however, instead of genomic deletions, mutations that introduce aberrant polyadenylation signals have been reported. We introduced two such patient specific mutations, described in MCL (Wiestner et al., 2007), specifically a point insertion of adenosine (A) between nucleotides number 308 and 309 after the stop codon of cyclin D1 or a deletion of trinucleotide sequence TCA at 309-311 of the 3'UTR in the expression construct with the full length cyclin D1 cDNA (**Fig.4.1a**). Indeed, upon introduction of these mutations cyclin D1 expression was significantly increased (1.33±0.051 fold in Ins308 and 1.31±0.046 fold in Δ309-311) compared to full length cyclin D1, (n=3, P=0.007 for Ins308 and P=0.009 for Δ309-311). Although the increase in expression was statistically significant, the increase was much more marked in the 3'UTR contruct. These data showed that these mutations could contribute to cyclin D1 overexpression in these patients (**Fig.4.1d**).

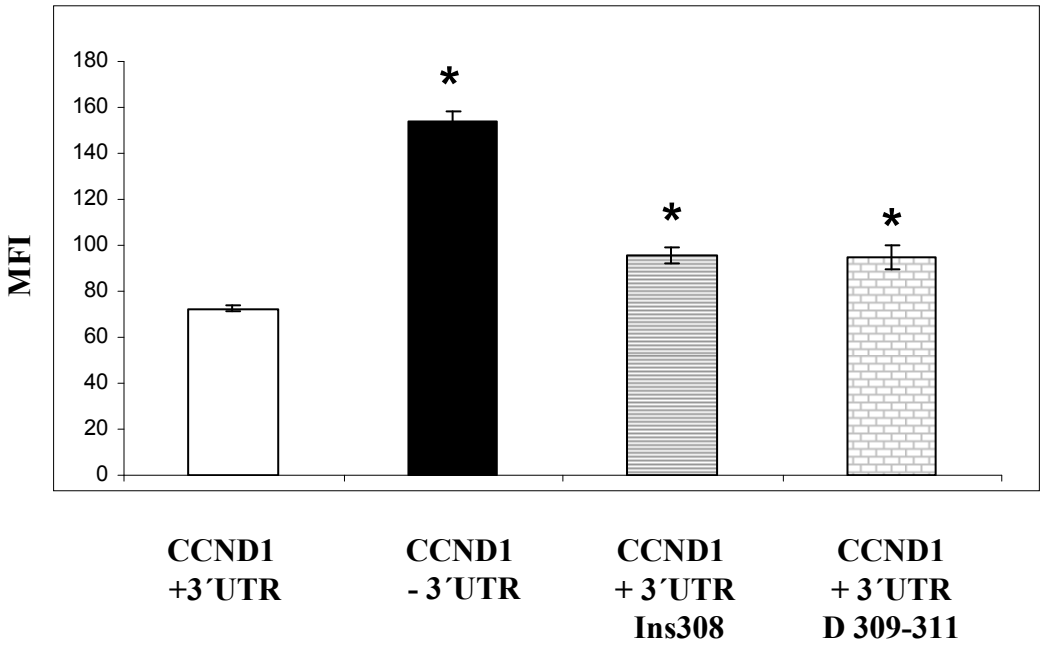


Fig.4.1d Increase in Cyclin D1 protein expression with the different 3'UTR mutations. A bar graph of mean fluorescence intensity of YFP calculated by flow cytometry. CCND1 + 3´UTR Ins308 and CCND1 + 3´UTR Δ 309-311 denote 3'UTR mutations observed in MCL patient samples. MFI: mean fluorescence intensity; * P<0.05 in each case.

4.2 The cyclin D1 3'UTR can regulate chimeric reporter activity:

Luciferase assays, due to a more accurate normalization of activity by the use of the dual luciferase reporter system, offer a more quantitative method for studying UTR mediated regulation (Chiu et al., 2005; Duan and Jefcoate, 2007). Therefore, and also in order to study the regulatory effect of the 3'UTR in isolation from the full length *cyclin D1* mRNA, we fused the cyclin D1 3'UTR to a constitutively expressed *luciferase* reporter. Normalized luciferase activity was reduced to an average of 22.8 % in HeLa cells with the addition of the long CCND1 3'UTR as compared to luciferase only (Luc) (**Fig.4.2a**). This effect was cell line independent as similar results were obtained in different cell lines such as A549 and MCF-7(**Fig.4.2b**). These data confirm the role of the full length cyclin D1 3'UTR in the regulation of cyclin D1 and demonstrate that the 3'UTR can also regulate chimeric reporter expression.

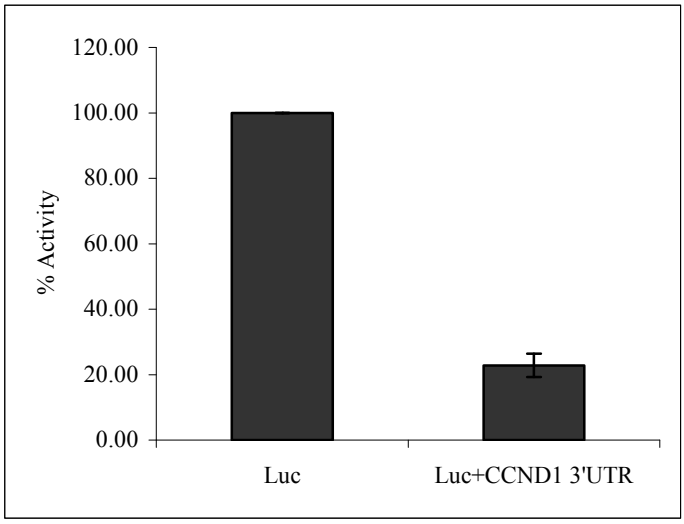


Fig.4.2a The 3'UTR of cylinD1 can regulate chimeric reporter activity. Percent normalized luciferase activity of a constitutively expressed luciferase reporter with or without the 3'UTR are presented confirming the regulatory potential of this region in isolation from cyclin D1. Normalized luciferase activity without the 3'UTR is considered 100 %.

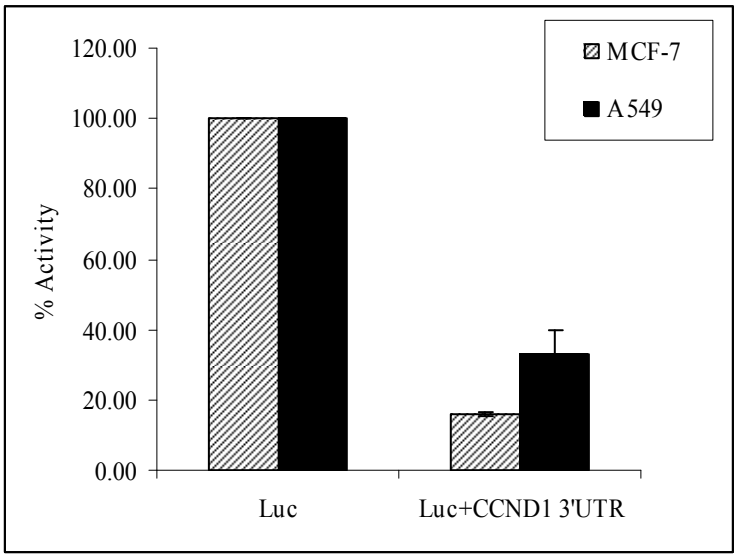


Fig.4.2b Regulation of chimeric reporter gene by Cyclin D1 3'UTR is independent of the cell line. The percent normalized luciferase activity is reduced to 15.82% and 32.92% in MCF-7 and A549 cell lines respectively.

4.3 Effect of the 3'UTR deletion on cyclin D1 function:

4.3.1 Effect on the proliferation of fibroblasts:

In order to check the effect of Cyclin D1 with and without 3'UTR on cell proliferation, NIH3T3 and IEC-18 cells were transduced with Cyclin D1constructs in the pMSCV-IRES-GFP vector (pMIG). GFP positive cells were checked for Cyclin D1 protein expression by western blotting using mouse anti-human cyclin D1 antibody.

Cell proliferation assays showed that the NIH3T3 cell line expressing *CCND1* without the 3'UTR showed a higher rate of proliferation (1.73 ± 0.64 fold after 8 days) (n=3, $P \leq 0.038$) compared to the cell lines with full 3'UTR (**Fig.4.3a left panel**). There was a remarkable difference in cell proliferation rate within the cell lines from day 4. The cells expressing *CCND1* without the 3'UTR proliferated more than the control cells expressing the empty pMIG vector. Thus, overexpression of *cyclin D1* on account of 3'UTR deletions in mouse fibroblasts might have increased the rates of G0-S phase and G1-S phase transition leading to increased proliferation rate (Quelle et al., 1993). These data demonstrate that the proliferative activity of cyclin D1 is dependent on the presence of its 3'UTR suggesting that mutations in this region which might increase the cyclin D1 protein expression as observed in **Fig.4.1d** could contribute to malignancy.

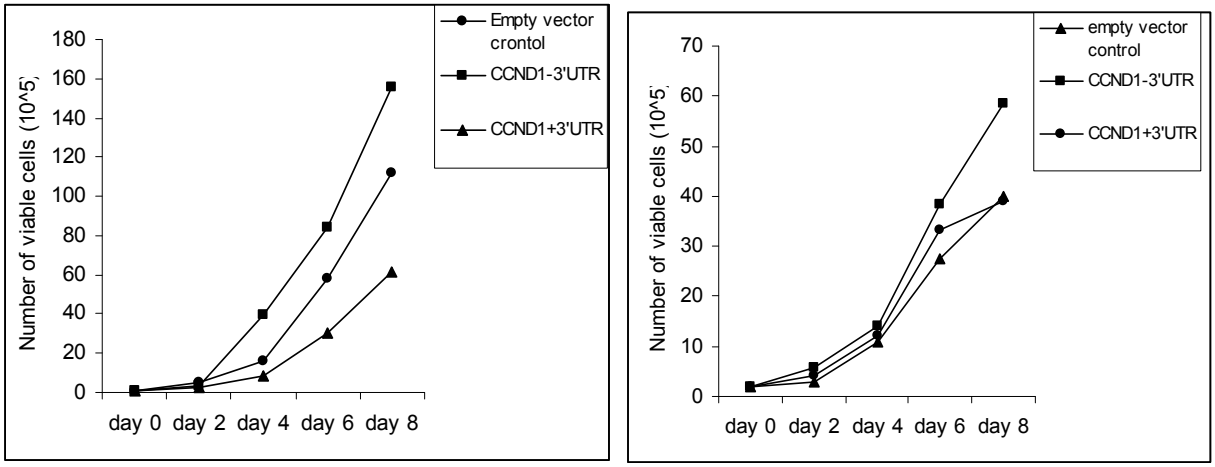


Fig.4.3a Proliferation curves of NIH3T3 and IEC-18 expressing the c*yclin D1* with or without 3'UTR or empty vector (*pMIG)*. This is a representative of the three independent experiments of cell proliferation. Number of viable cells was determined after trypan blue exclusion.

The proliferation assay was also performed in the IEC-18, the rat epithelial cell line which normally does not express *cyclin D1*. We made IEC18 cell lines which stably expressed *cyclin D1* with or without 3'UTR (**Fig.4.3a right panel**). The cell line expressing *cyclin D1* without the 3'UTR showed a higher proliferation rate (1.49 fold after 8 days) than the cells expressing CCND1 with 3'UTR or empty vector, similar to NIH3T3 cell lines. However, in the IEC18 cell lines, cells expressing CCND1 with the 3'UTR or empty vector transduced cells showed comparable rates of proliferation.

4.3.2 Effects on cell cycle regulation:

Since cyclin D1 is a G1 type of cyclin that plays an important role in cell cycle regulation it was interesting to study effect of the cyclin D1 3'UTR on cell cycle in IEC-18 cell line which does not express *cyclin D1* endognously. IEC-18 cell lines were thus transduced with cyclin D1 with or without 3'UTR or empty vector as discussed in methods section to stably express *cyclin D1* and cell cycle analysis was performed. There was no change in percentage of cells in G1 and G2 phases of cell cycle in cells expressing CCND1 with 3'UTR or empty vector as compared to the untransduced control IEC-18 cells (Table 1). However cells expressing CCND1 without

3'UTR showed a significant reduction in percentage of cells in G1 phase and increase in percentage of cells in G2 phase.

Type of cells	G1 phase	S phase	G2 phase
Conrol cells (untransduced)	68	26.75	5.26
Empty vector control	67.14	24.26	8.61
Cyclin D1 + 3'UTR	70.43	22.12	7.45
Cyclin D1- 3'UTR	57.84	21.9	20.26

Table 1 The above table indicates the percantage of cells in different phases of cell cycle in untransduced IEC-18 cell line and stable cell lines expressing empty vector, cyclin D1 with or without 3'UTR.

Increase in G2 phase demonstrates that the cells expressing *cyclin D1* without the 3'UTR proliferate with shorter G1-S phase as compared to that with cells with cyclin D1 with 3'UTR or control cells (**Fig.4.3b**). The change in cell cycle pattern in IEC-18 cell stable cell lines confirms the cell proliferation data showing the potential role of the 3'UTR in cyclin D1 regulation.

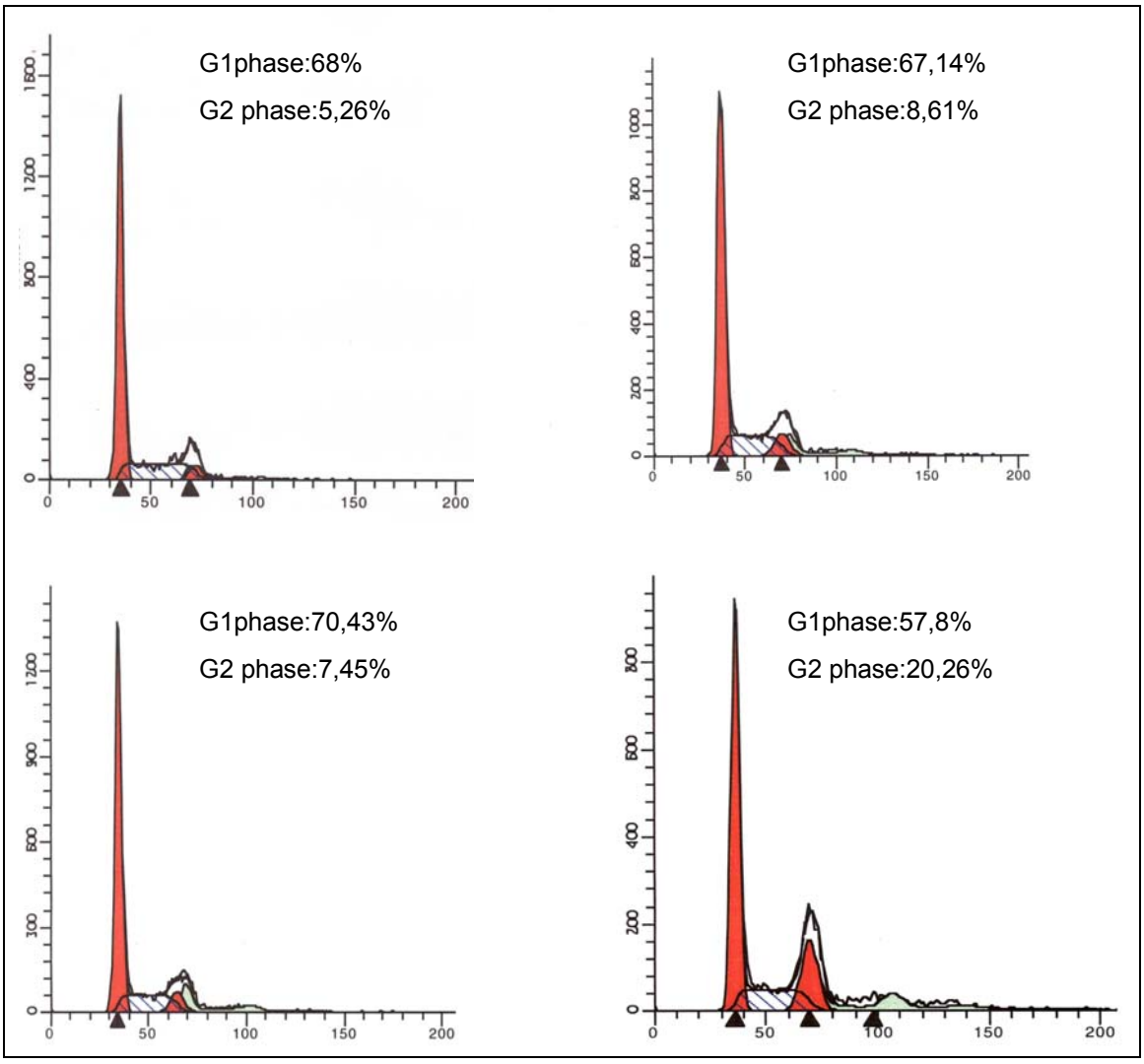


Fig.4.3b Cell cycle analysis of IEC-18 cell lines. The upper panel indicates control cells on left and IEC-18 with empty vector on right. The lower panel shows the IEC-18 cells with CCND1 with 3'UTR on left and CCND1 without 3'UTR on right. Two red peaks in the figure indicate the G1 (left) and the G2 phase (right) of cell cycle. This figure is representative of three independent experiments.

Our data shows the regulatory potential of the cylin D1 UTR and its impact on cyclin D1 expression and function. These data indicate the presence of elements in the 3'UTR of cyclin D1 that regulate cyclin D1 expression and function.

4.4 Bioinformatic analysis of cyclin D1 3'UTR:

Identification of ARE elements:

We scanned the 3'UTR for elements that could potentially regulate cyclin D1 expression. An analysis for the ARE sequences was performed by the University of California Santa Cruz Genome Browser May 2004 (hg17) assembly. We observed that the UTR contains 7 reiterations of the signature ARE sequence AUUUA. 6 of these 7 motifs can be found in an approximately 474 nt AU-rich region between nucleotides 1369 and 1838 of the cyclin D1 3'UTR.

GGGCGCCAGGCAGGCGGGCGCCACCGCCACCCGCAGCGAGGGCGGAGCCGGCCCCAGGUGCUCCCCUGACAGUCCCUCCUCUCCGGAGCAUUUUGAU
ACCAGAAGGGAAAGCUUCAUUCUCCUUGUUGUUGGUUGUUUUUUCCUUUGCUCUUUCCCCCUUCCAUCUCUGACUUAAGCAAAAGAAAAAGAUUACC
CAAAAACUGUCUUUAAAAGAGAGAGAGAGAAAAAAAAAAUAGUAUUUGCAUAACCCUGAGCGGUGGGGGGAGGAGGGUUGUGCUACAGAUGAUAGAGG
AUUUUAUACCCCAAUAAUCAACUCGUUUUUAUAUUAAUGUACUUGUUUCUCUGUUGUAAGAAUAGGCAUUAACACAAAGGAGGCGUCUCGGGAGAGG
AUUAGGUUCCAUCCUUUACGUGUUUAAAAAAAAGCAUAAAAACAUUUUAAAAACAUAGAAAAAUUCAGCAAACCAUUUUUAAAGUAGAAGAGGGUUU
UAGGUAGAAAAACAUAUUCUUGUGCUUUUCCUGAUAAAGCACAGCUGUAGUGGGGUUCUAGGCAUCUCUGUACUUUGCUUGCUCAUAUGCAUGUAGU
CACUUUAUAAGUCAUUGUAUGUUAUUAUAUUCCGUAGGUAGAUGUGUAACCUCUUCACCUUAUUCAUGGCUGAAGUCACCUCUUGGUUACAGUAGCG
UAGCGUGCCCGUGUGCAUGUCCUUUGCGCCUGUGACCACCACCCCAACAAACCAUCCAGUGACAAACCAUCCAGUGGAGGUUUGUCGGGCACCAGCC
AGCGUAGCAGGGUCGGGAAAGGCCACCUGUCCCACUCCUACGAUACGCUACUAUAAAGAGAAGACGAAAUAGUGACAUAAUAUAUUCUAUUUUUAUA
CUCUUCCUAUUUUUGUAGUGACCUGUUUAUGAGAUGCUGGUUUUCUACCCAACGGCCCUGCAGCCAGCUCACGUCCAGGUUCAACCCACAGCUACUU
GGUUUGUGUUCUUCUUCAUAUUCUAAAACCAUUCCAUUUCCAAGCACUUUCAGUCCAAUAGGUGUAGGAAAUAGCGCUGUUUUUGUUGUGUGUGCAG
GGAGGGCAGUUUUCUAAUGGAAUGGUUUGGGAAUAUCCAUGUACUUGUUUGCAAGCAGGACUUUGAGGCAAGUGUGGGCCACUGUGGUGGCAGUGGA
GGUGGGGUGUUUGGGAGGCUGCGUGCCAGUCAAGAAGAAAAAGGUUUGCAUUCUCACAUUGCCAGGAUGAUAAGUUCCUUUCCUUUUCUUUAAAGAA
GUUGAAGUUUAGGAAUCCUUUGGUGCCAACUGGUGUUUGAAAGUAGGGACCUCAGAGGUUUACCUAGAGAACAGGUGGUUUUUAAGGGUUAUCUUAG
AUGUUUCACACCGGAAGGUUUUUAAACACUAAAAUAUAUA**AUUUA**UAGUUAAGGCUAAAAAGUAU**AUUUA**UUGCAGAGGAUGUUCAUAAGGCCAGUA
UG**AUUUA**UAAAUGCAAUCUCCCCUUG**AUUUA**AACACACAGAUACACACACACACACACACACACACAAACCUUCUGCCUUUGAUGUUACAG**AUUUA**A
UACAGUUUAUUUUUAAAGAUAGAUCCUUUUAUAGGUGAGAAAAAAACAAUCUGGAAGAAAAAAACCACACAAAGACAUUGAUUCAGCCUGUUUGGCG
UUUCCCAGAGUCAUCUGAUUGGACAGGCAUGGGUGCAAGGAAAAUUAGGGUACUCAACCUAAGUUCGGUUCCGAUGAAUUCUUAUCCCCUGCCCCUU
CCUUUAAAAAACUUAGUGACAAAAUAGACAAUUUGCACAUCUUGGCUAUGUAAUUCUUGUAAUUUUU**AUUUA**GGAAGUGUUGAAGGGAGGUGGCAAG
AGUGUGGAGGCUGACGUGUGAGGGAGGACAGGCGGGAGGAGGUGUGAGGAGGAGGCUCCCGAGGGGAAGGGGCGGUGCCCACACCGGGGACAGGCCG
CAGCUCCAUUUUCUUAUUGCGCUGCUACCGUUGACUUCCAGGCACGGUUUGGAAAUAUUCACAUCGCUUCUGUGUAUCUCUUUCACAUUGUUUGCUG
CUAUUGGAGGAUCAGUUUUUUGUUUUACAAUGUCAUAUACUGCCAUGUACUAGUUUUAGUUUUCUCUUAGAACAUUGUAUUACAGAUGCCUUUUUUG
UAGUUUUUUUUUUUUUUAUGUGAUCAAUUUUGACUUAAUGUGAUUACUGCUCUAUUCCAAAAAGGUUGCUGUUUCACAAUACCUCAUGCUUCACUUA
GCCAUGGUGGACCCAGCGGGCAGGUUCUGCCUGCUUUGGCGGGCAGACACGCGGGCGCGAUCCCACACAGGCUGGCGGGGGCCGGCCCCGAGGCCGC
GUGCGUGAGAACCGCGCCGGUGUCCCCAGAGACCAGGCUGUGUCCCUCUUCUCUUCCCUGCGCCUGUGAUGCUGGGCACUUCAUCUGAUCGGGGGCG
UAGCAUCAUAGUAGUUUUUACAGCUGUGUUAUUCUUUGCGUGUAGCUAUGGAAGUUGCAUAAUUAUUAUUAUUAUUAUAACAAGUGUGUCUUAC
GUGCCACCACGGCGUUGUACCUGUAGGACUCUCAUUCGGGAUGAUUGGAAUAGCUUCUGGAAUUUGUUCAAGUUUUGGGUAUGUUUAAUCUGUUAUG
UACUAGUGUUCUGUUUGUUAUUGUUUUGUUAAUUACACCAUAAUGCUA**AUUUA**AAGAGACUCCAAAUCUCAAUGAAGCCAGCUCACAGUGCUGUGUG
CCCCGGUCACCUAGCAAGCUGCCGAACCAAAAGAAUUUGCACCCCGCUGCGGGCCCACGUGGUUGGGGCCCUGCCCUGGCAGGGUCAUCCUGUGCUC
GGAGGCCAUCUCGGGCACAGGCCCACCCCGCCCCACCCCUCCAGAACACGGCUCACGCUUACCUCAACCAUCCUGGCUGCGGCGUCUGUCUGAACCA
CGCGGGGGCCUUGAGGGACGCUUUGUCUGUCGUGAUGGGGCAAGGGCACAAGUCCUGGAUGUUGUGUGUAUCGAGAGGCCAAAGGCUGGUGGCAAGU

GCACGGGGCACAGCGGAGUCUGUCCUGUGACGCGCAAGUCUGAGGGUCUGGGCGGCGGGCGGCUGGGUCUGUGCAUUUCUGGUUGCACCGCGGCGCU
UCCCAGCACCAACAUGUAACCGGCAUGUUUCCAGCAGAAGACAAAAAGACAAACAUGAAAGUCUAGAAAUAAAACUGGUAAAACCCCA

Fig.4.4a Identification of AU-rich elements in the cylin D1 3'UTR. AUUUA pentamers identified in the cyclin D1 3'UTR are highlighted in gray and the 474 nt. region deleted in the ΔARE-YFP constructs is underlined. In the Flu-7ΔARE construct, all AUUUA sites were mutated to ACUGA.

Identification of potential microRNA binding sites:

An analysis of the cyclin D1 3'UTR for putative microRNA binding sites was performed using microRNA prediction software mentioned in section 2.5. These results indicated that the 3.1 kb cyclin D1 3'UTR is a potential target of several microRNAs. The miRNAs predicted to target cyclin D1 3'UTR include let-7 family members, miR-17-92 cluster, miR-1/206 and miR-15/16 family members. All of these microRNAs are shown to have conserved binding sites across various species. The microRNAs themselves are conserved across species too. miR-15/16 microRNAs are predicted to have two highly conserved binding sites at nucleotides position 1961-1967 and 2034-2040 whereas miR-17-92 cluster miRNAs have a conserved putative target site at nucleotide position 1014- 1020 (**Fig.4.4b**). Free energy values for miR-15/16 and miR-17 cluster miRNAs are -21.5 and -19.5 respectively which show the high possibility of targeting the cyclin D1 3'UTR.

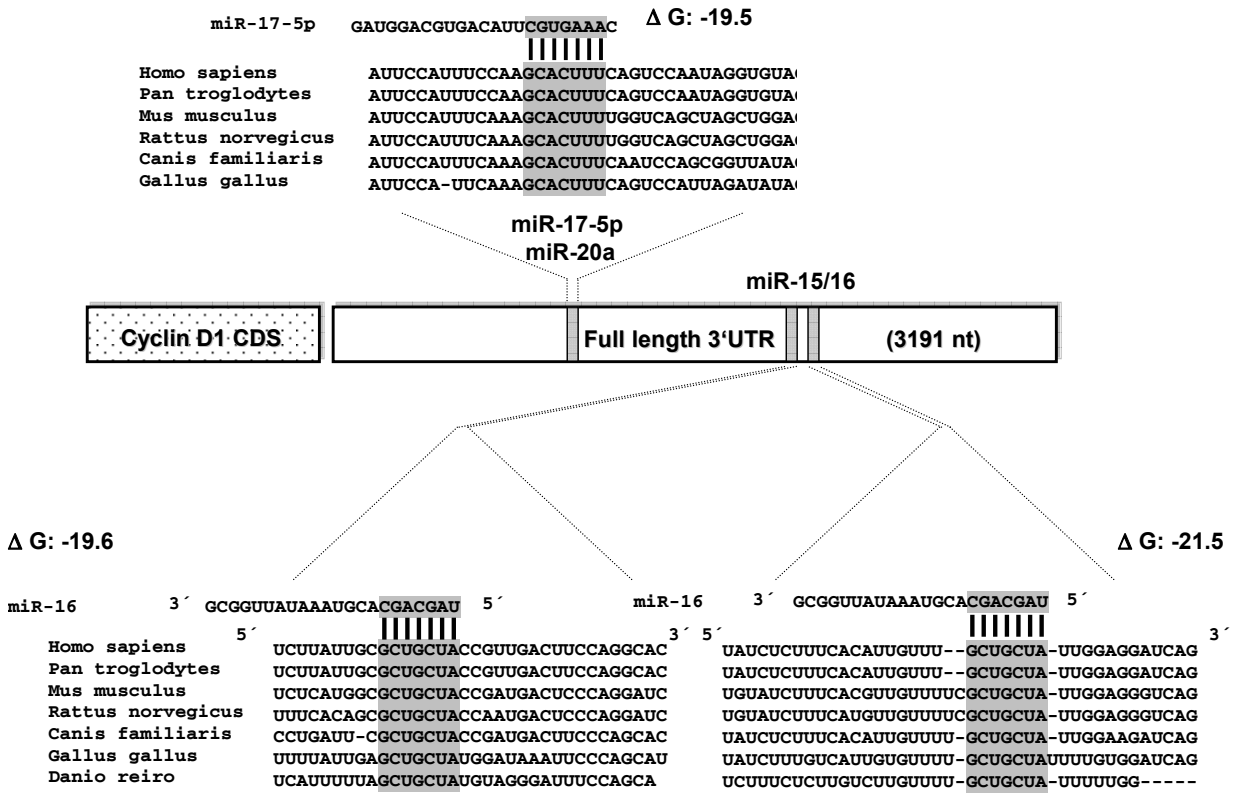


Fig.4.4b The microRNA binding sites in cyclin D1 3'UTR are highly conserved across species The cyclin D1 3'UTR shows two highly conserved target sites (seven species) for miR-15/16 family at nucleotides position 1961-1967 and 2034-2040 and one highly conserved (six species) site for miR-17/20a at position position 1014-1020. The conservation of the seed sequence of the microRNA and its complementary region across species is depicted (nucleotides highlighted in gray) and the minimum free energy values of miRNA-target interaction are included as ΔG values. Note: The figure is illustrative and not drawn to scale.

Interestingly the ARE containing regions and the potential microRNA regulatory regions in the 3'UTR are non-overlapping (**Fig.4.4c**) indicating independent functions of these regions in cyclin D1 regulation.

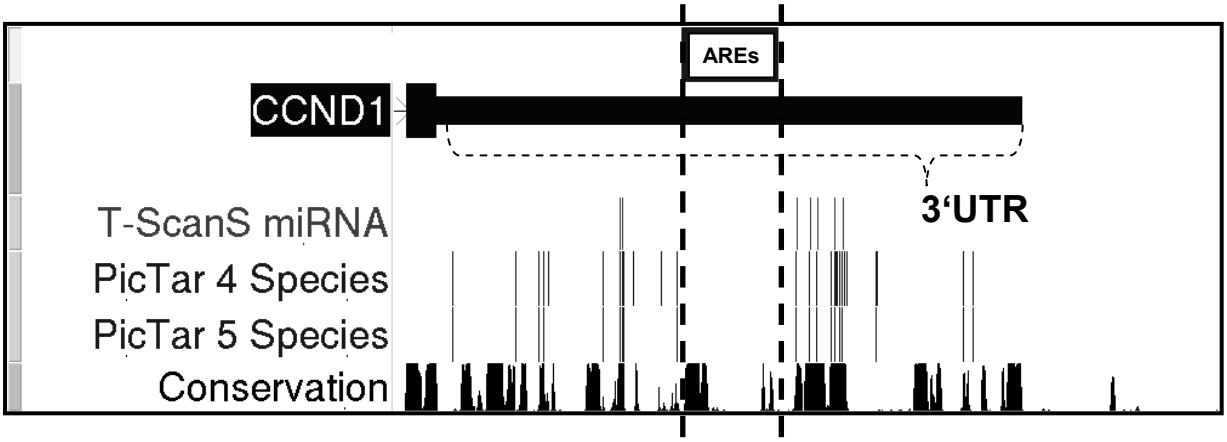


Fig.4.4c Different non-overlapping potential regulatory regions in the cyclin D1 3'UTR: Analysis of the cyclin D1 3'UTR using different microRNA target prediction software shows several potential microRNA binding sites shown in vertical lines below the cyclin D1 3'UTR. The AU rich elements (AREs) which include several highly conserved AUUUA motifs are contained within an approximately 467 nt region in the UTR shown in a closed rectangle above the UTR. MicroRNA target regions using the TargetScan software (labelled T-ScanS miRNA) and PicTar software depicting microRNAs conserved in 4 or 5 species are labelled as PicTar 4 species and PicTar 5 species respectively. Each microRNA binding site is shown as a vertical line below the 3'UTR. Cross species conservation is depicted at the bottom of the figure

4.5 The repressive function of the cyclin D1 3'UTR is independent of AREs:

AREs in the cyclin D1 3'UTR have been previously reported, but a study of their contribution to cyclin D1 expression remained to be performed. It has been hypothesised that cyclin D1 AREs are responsible for its regulation and that the loss of these potentially destabilizing elements from the cyclin D1 UTR could contribute to the observed stable overexpression of cyclin D1 although this possibility has not yet been addressed experimentally. We therefore deleted a 474 nt region of the cyclin D1 3'UTR which includes 6 out of the 7 AUUUA pentamers (nucleotides 1359 to 1832 of the 3'UTR) in the full-length cyclin D1 (ΔARE-YFP) construct. Transfection of the ARE deletion construct, failed to show an increase in cyclin D1 expression. In contrast, there was a modest (0.77±0.08 fold, n=3) but significant reduction (P=0.038) of cyclin D1 protein expression measured by quantitative FACS analysis (**Fig.4.5a**) suggesting that the AREs might have a stabilizing function on *cyclin D1* mRNA.

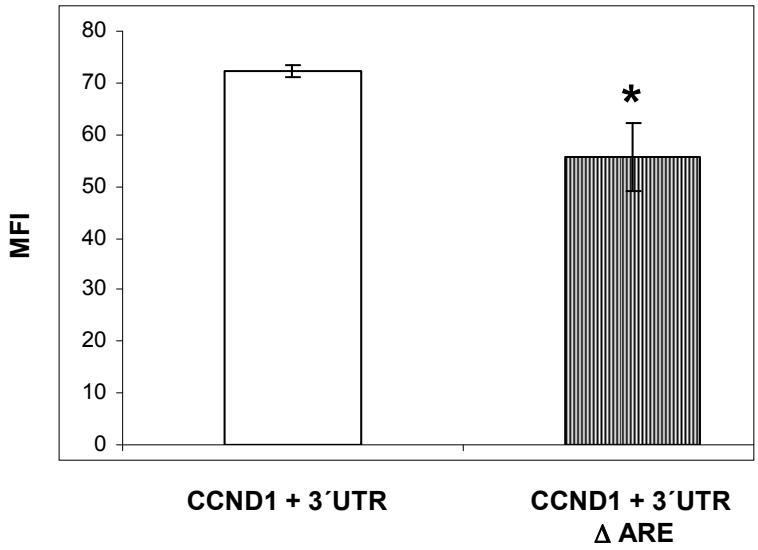


Fig.4.5a The mean fluorescence intensity of YFP expression using cylinD1-YFP with the full UTR is compared with the cyclin D1-YFP with the 3'UTR harbouring deletions of a 474 nt region which includes an AU rich element (ARE) region interspersed with 6 of the 7 AUUUA motifs present in the cyclin D1 3'UTR CCND1 + 3´UTR ΔARE. MFI: mean fluorescence intensity; * P<0.05

This is interesting because AREs in the cyclin D1 have been hypothesised to play a destabilizing role.

Since the luciferase assay is much more sensitive in measuring the impact of these changes, we sought to assess the effects of ARE deletion on luciferase reporter activity. We therefore systematically mutated all seven AUUUA pentamers from the cyclin D1 3'UTR fused to a luciferase reporter to 'ACUGA'. As we have mentioned previously, the addition of the 3'UTR to the luciferase reported leads to a significant reduction in luciferase activity. If the ARE elements have a destabilizing effect on the *cyclin D1* mRNA as has been hypothesised (Rimokh et al., 1994a; Wiestner et al., 2007), deletion of these signature ARE sequences should rescue the reduction in luciferase activity by the 3'UTR. In contrast, the luciferase activity is drastically reduced to 3.24 % ± 3.73 compared to that with the full length 3'UTR with intact ARE sequences (**Fig.4.5b**) in HeLa cells.

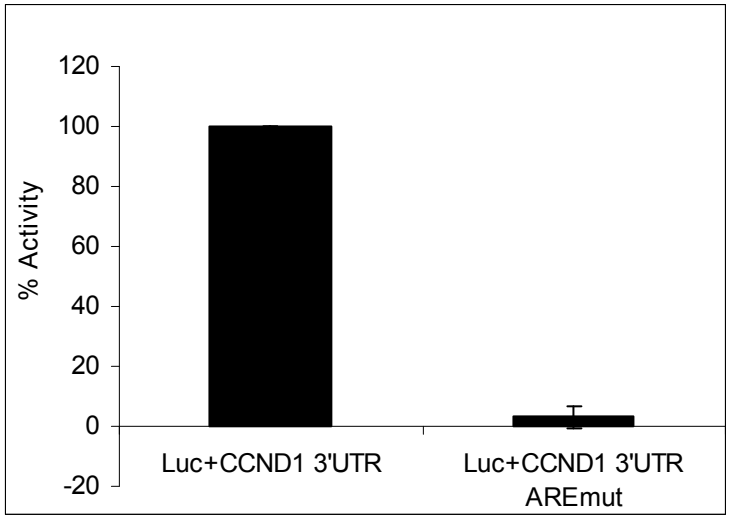


Fig.4.5b The normalized luciferase activity is reduced to 3.24% upon mutation of the 7 ARE sequences in the cyclin D1 3'UTR when compared to the full 3'UTR

FACS analysis and luciferase reporter assay results together suggest that these AREs have a stabilizing effect on *cyclin D1* transcripts similar to other recently reported AREs (Fialcowitz-White et al., 2007). It can be inferred from these data that the increased level of cyclin D1 expression upon deletion of the 3'UTR is likely to be attributable to other regulatory elements.

4.6 The 3'UTR of cyclin D1 is a target of microRNA mediated regulation:

Other regulatory elements analysed are the predicted miRNAs that might target the cyclin D1 3'UTR and finetune the cyclin D1 protein expression. One of the most interesting of these microRNAs were microRNAs of the miR-15/16 family and the miR-17 cluster, both of which have been shown to be involved in lymphoid malignancies. Moreover, the miR15/16 microRNAs have been shown to regulate the expression of cell cycle regulatory genes. MicroRNAs of the miR-17 cluster are located in a region of DNA which is amplified in a number of human B cell-lymphomas. Recently, the amplification of this cluster has also been demonstrated in a mantle cell lymphoma cell line (Rinaldi et al., 2007). Several putative microRNA targets have been

predicted by computational methods, with a very minor proportion being experimentally validated.

4.6.1 Cyclin D1 is the direct target of miR-17 cluster miRNAs:

We sought to determine whether miR-17 cluster miRNAs, which have been shown to be amplified in B cell lymphomas including MCL, target cyclin D1. The dual-luciferase reporter assay was employed to show the direct interaction between miR-17/20a miRNAs and the 3'UTR of Cyclin D1. First of all, the luciferase assay was performed to check the functionality of the miR-17-92 miRNAs cloned into the CMV-17-cluster construct in a cell line. The miR-17-5p and miR-20a sensor plasmids (see materials and methods) that contain two repeats of the perfect complementary sequence to the miR-17-5p and miR-20a binding sites respectively were co-transfected with CMV-17-cluster into A549 cells. Similar transfections were performed with pGL3-Control (Luc) empty vector as a control. The normalised luciferase activity in percent with sensor plasmids was compared to that with the Luc vector.

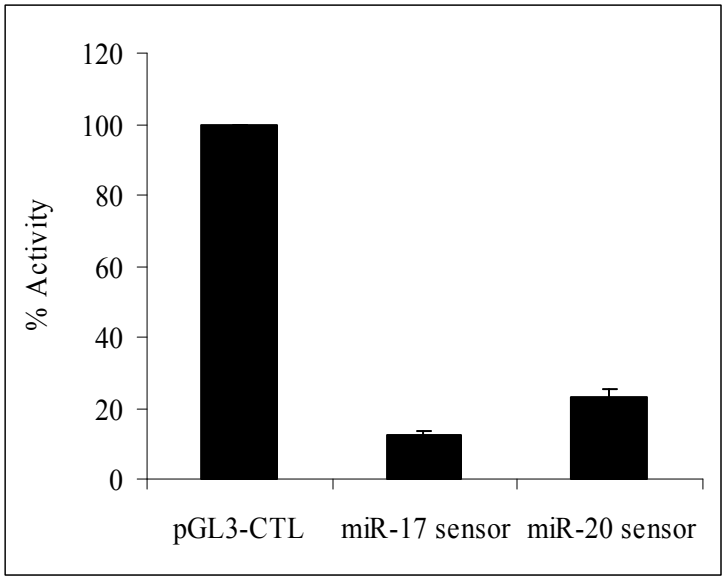


Fig.4.6a The CMV-17-cluster is functional in Dual-Luciferase Assay System. The luciferase activity was normalized to Renilla luciferase and the normalized percent luciferase activities with sensor plasmids were compared to that with the Luc. The experiments are performed in triplicates and the ±S.E.M. values are shown in the graph.

The luciferase activity significantly reduced to 12.52% and 23.14% with miR-17-5p and miR-20a sensor plasmids respectively confirmed the functionality of the CMV-17-cluster construct.

After the proof of functionality of CMV-17-cluster plasmid, the luciferase reporter assay was performed with the reg-17-92 and reg-17-92-M constructs (see materials and methods) in A549 cells to show the direct interaction between 3'UTR::miR-17-5p/miR-20a.

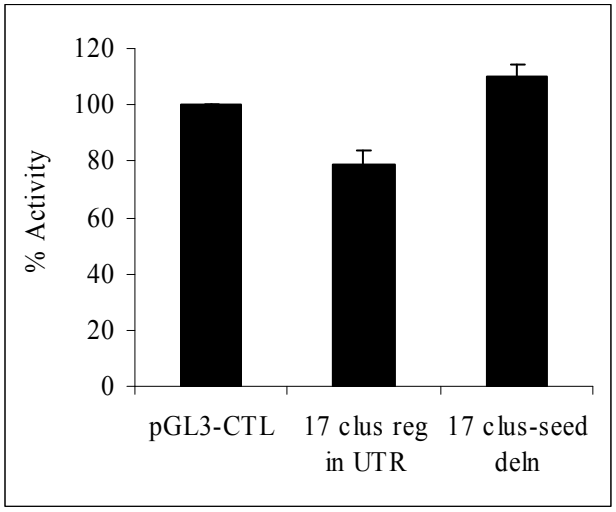


Fig.4.6b Specific targeting of cyclin D1 by miR-17 cluster microRNAs. The normalised luciferase activity after transfection of the constitutively expressed luciferase plasmid is compared to the luc-reg-17/20a or the luc-17/20a mut constructs. The activity of the luciferase without a 3'UTR is considered 100 %. The assay is performed in triplicates and ±S.E.M. values of three independent experiments are shown.

We observed that the introduction of a region containing a highly conserved miR-17/20a binding site (reg-17-92) led to a significant reduction in the mean normalized luciferase activity by an average of 21.46%±5.44 in transfected cells upon co-transfection with 1.5µg of the CMV-miR-17 cluster plasmid. This reduction was completely rescued by the mutation of the putative conserved 'seed' sequence in the miRNA binding sites (reg-17-92-M) confirming that microRNAs of the miR-17 cluster play a role in the regulation of cyclin D1.

4.6.2 MicroRNAs miR-15a and miR-16-1 specifically target the cyclin D1:

In order to directly test whether the cyclin D1 3'UTR is a direct target of the mir-15/16 microRNAs, we cloned a small portion of the region containing putative miR-15/16 binding sites, reg-15/16 (see Materials and Methods) harbouring both the potential miR-15/16 binding sites was cloned downstream of Luc and used for the reporter assay. For the analysis of miR-15/16::cyclin D1 interaction, synthetic oligonucleotide mimics of microRNA precursors (Pre-miRs) for mir-16-1 and miR-15a or a non-cognate microRNA control (miR-95) (see Materials and Methods) were transfected separately in addition to the reg-15/16 reporter construct into HeLa cells and their normalized luciferase activity was compared to the reg-15/16 reporter activity without exogeneously introduced microRNAs after 24 hours. Similar transfections were also performed with a construct (reg-15/16-M3) in which mutations were introduced in the region with perfect complementarity, termed the 'seed' sequence to determine the specificity of the interaction.

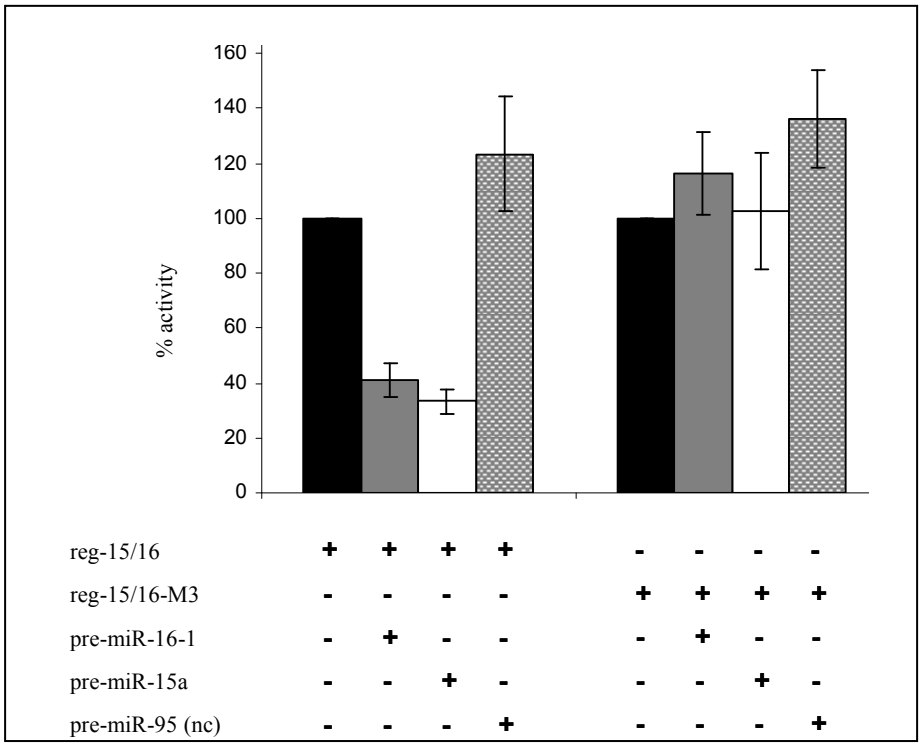


Fig.4.6c The cyclin D1 3'UTR is a target of miR-15/16 family microRNAs. The normalised luciferase activity after transfection of the luciferase plasmid in which a region containing the two miR-15/16 binding sites (luc-reg-15/16) was cloned with or without the addition of miR-15a, miR-16-1 or miR-95 non-cognate microRNAs is shown on the left side of the panel. On the right side, luciferase activity using the same region with

mutations in the 'seed' sequence of these microRNA binding sites (reg-15/16) with or without externally added microRNAs is shown.

As expected the normalized luciferase activity of reg-15/16 was reduced significantly by 58.99%±6.12 and 66.73%±4.44 upon addition of miR-16-1 and miR-15a precursors respectively whereas the non-cognate control miR-95 did not show such a reduction, showing that the miR15/16 microRNAs specifically target the cyclin D1 3'UTR. Moreover, the reg-15/16-M3 plasmid was insensitive to miR15/16 introduction confirming the specificity of the microRNA::target interaction (**Fig.4.6c**). The dual-luciferase reporter assays demonstrated that the Cyclin D1 3'UTR is directly and specifically targeted by the microRNAs of miR-17 cluster and miR-15/16 family as both of these family members reduced the luciferase activity significantly. Even though the reduction with miR-17 microRNAs was significant, it was not as high as that with the miR-15/16 miRNAs, probably because there are two putative miR-15/16 binding sites in reg-15/16 as opposed to a single miR-17/20a binding site in reg-17-92. These data show that the binding of these microRNAs play an important role in the repression of cyclin D1 mediated by the 3'UTR and that the abrogation of microRNA regulation by aberrant mutation or deletion of these regions could result in cyclin D1 overexpression observed in human tumors including MCL.

4.6.3 Effect of miR-17-92 and miR15/16 miRNAs on endogenous cyclin D1:

Although luciferase reporter assays demonstrate direct interactions of microRNAs with the cyclin D1, it is important to demonstrate the targeting of endogenous *cyclin D1* by these microRNAs. Moreover, it is necessary to demonstrate that cyclin D1 expression is unaffected by these microRNAs in cells in which the cyclin D1 3'UTR has been deleted. Since cyclin D1 overexpression is also common in breast cancer and since MCL cell lines are known to be difficult to transfect, we used the cyclin D1 overexpressing cell lines MCF-7 and MDA-MB-453. The MCF-7 cell line harbors an intact cyclin D1 gene whereas the MDA-MB-453 cell line harbors genomic deletions of the cyclin D1 3'UTR (Lebwohl et al., 1994). Upon transfection of these cell lines with vectors expressing the miR-17 cluster microRNAs (CMV-miR-17 cluster) or

the miR-15/16 family (a kind gift from Dr Patrick Lewis, Merck Co. Inc.), we observed that the levels of endogeneous cyclin D1 protein could be significantly downregulated in the MCF-7 cell line but not in the MDA-MB-453 cell line which has cyclin D1 3'UTR deletions (**Fig.4.6d**).

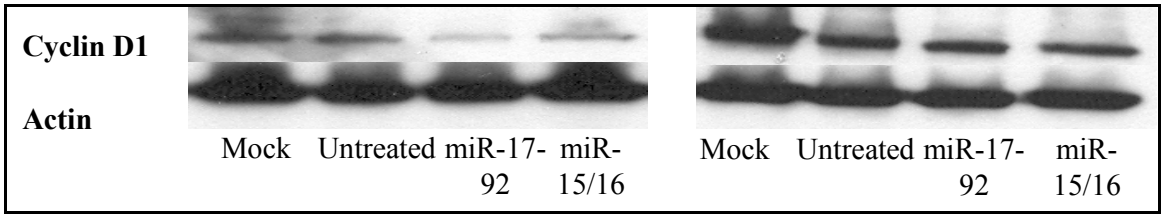


Fig.4.6d Western blots show the levels of endogenous cyclin D1 levels after mock trasnfections, no trasnfections or trasnfections with miR-17 cluster and miR15/16 microRNA expressing plasmids in MCF-7 (Left panel) and MDA-MB453 (right panel) cell lines. This blot is representative of three independent experiments. Cyclin D1 protein expression is normalized to actin

Significant decrease in cyclin D1 protein expression in MCF-7 cells indicated that externally introduced miR-17 cluster and miR-15/16 family miRNAs bind to the 3'UTR of cyclin D1 and negatively regulate the *cyclin D1* mRNA. No change in cyclin D1 protein expression in MDA-MB-453 cells demonstrated that loss of the 3'UTR could help cyclin D1 escape the repressive effect of the miRNAs. Thus, overexpression of cyclin D1 in many tumors might partly be due to its escape from the miRNA binding to its 3'UTR. Further mRNA expression analysis of *cyclin D1* in both of these cell lines was performed and interestingly, in both cases, the levels of mRNA were unchanged, showing that these microRNAs mediate post-transcriptional regulation of cyclin D1 (**Fig.4.6e**).

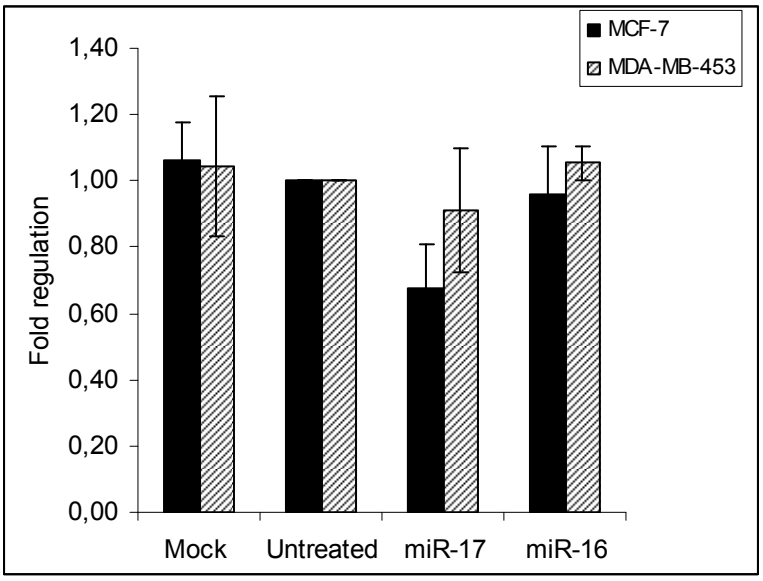


Fig.4.6e *Cyclin D1* mRNA expression by Taqman™ real time RT-PCR. The graph indicates the mean of three independent experiments and ±S.E.M. values. Relative expression of *cyclin D1* mRNA was normalized to the housekeeping TBP gene.

Cyclin D1 mRNA expression was insignicantly lowered upon introduction of miR-17 cluster miRNAs in MCF-7 cells whereas no change was observed with miR-15/16 miRNAs in both cell lines. These data show that the binding of these microRNAs play an important role in the repression of cyclin D1 mediated by the 3'UTR and that the abrogation of microRNA regulation by aberrant mutation or deletion of these regions render the cyclin D1 insensitive to microRNA mediated repression and account at least partially for cyclin D1 over-expression observed in human tumors.

Discussion

Many human tumor cells escape normal regulation of cell cycle progression and growth either by overexpressing cyclin D1 or by inactivating proteins that regulate it or are necessary for its activity (Sherr, 1996). However, mutations in the coding region of cyclin D1 have not been reported. The most common alteration involving the cyclin D1 locus is the t (11;14) translocation which can be observed in virtually all cases of MCL. Although the juxtaposition of the cyclin D1 gene to the IgH enhancer leads to the ectopic expression of cyclin D1 in MCL, this mechanism is thought to be insufficient for cyclin D1 overexpression, especially in the more proliferative tumor types. In addition, these hyperproliferative forms of MCL which are known to have a poor prognosis are associated with the expression of shorter *cyclin D1* transcripts. These transcripts express a truncated form of the 3'UTR, which was believed to result from a normal alternative splicing event. In 2006, Wiestner et al., demonstrated that the shorter *cyclin D1* transcripts derive from genomic mutations in the 3'UTR rather than from normal alternative splicing events (Wiestner et al., 2007). They observed that genomic deletions or point mutations that introduce premature polyadenylation signals lead to the truncation of this region in a number of MCL patients and cell lines. A systematic study of this region therefore was warranted to the pathogenesis of cyclin D1 deregulated tumors.

The 3' UTRs of eukaryotic mRNAs play important roles in post-transcriptional regulation by modulating transcript stability, translation efficiency, decay rate and subcellular localization (Mazumder et al., 2003). This region may therefore be viewed as a regulatory region that is important for the regulation of most genes. Interestingly, a recent study has shown that upon proliferation, several genes express truncated forms of their 3'UTRs by making use of proximal polyadenylation sites, thereby escaping 3'UTR mediated regulatory signals (Sandberg et al., 2008). Given their important regulatory role, mutations that affect this region have been implicated in various diseases and their pathological role is becoming increasingly apparent (Conne et al., 2000). The 3'UTR of the cyclin D1 gene is approximately 3.1 kb long, which is more than 3 times longer than the average length (1.02 kb) of human 3'UTRs (Pesole et al., 1997) suggesting a potential for 3'UTR mediated regulation of cyclin D1 expression. In this

dissertation, several regulatory elements were computationally identified in this region of the cyclin D1 gene. It is important to note that the disease specific forms of the *cyclin D1* transcript described in MCL lack these potential regulatory regions. Moreover, earlier studies have described deletions of this region in breast cancer as well as in neuroblastoma. Since the cyclin D1 gene is also deregulated in solid tumors, especially in breast cancer, a screening of the cyclin D1 3'UTR for mutations in these tumors warrants investigation.

Interestingly, given the crucial role of cyclin D1 in many different tumors, several studies have sought to establish *cyclin D1* overexpression models. Although some studies have shown that *cyclin D1* overexpression in fibroblasts has limited effects on cell growth and transformation, other reports demonstrated that cyclin D1 can transform fibroblasts (Asano et al., 1995; Jiang et al., 1993; Quelle et al., 1993). It has been hypothesized that these apparently contradictory results are due to the different expression levels of cyclin D1 in these various studies (Asano et al., 1995; Quelle et al., 1993). In our study, the expression of cyclin D1 in the NIH3T3 murine fibroblast cell line and in IEC-18 rat primary epithelial enterocytes, leads to an increase in proliferation, albeit only in cells transduced with 3'UTR deleted cyclin D1. Therefore, our observation that the phenotypic consequence of *cyclin D1* expression is different depending on the presence or absence of its 3'UTR is important and most probably of clinical relevance as it recapitulates the aforementioned study showing that states of proliferation of lymphocytes correlate with the expression of shortened 3'UTRs of several genes (Sandberg et al., 2008). It is important to note that in the natural setting, genes are normally flanked by several elements including 5 and 3' UTRs. In this physiological scenario, the expression of that gene would have functional consequences that are different from those observed in the artificial cell line setting in which these elements (such as the 3'UTR) are excluded. Therefore, even though cyclin D1 is overexpressed in several tumors, malignant changes in these regulatory elements could substantially enhance its contribution to transformation. The relatively poor prognosis of MCL with high levels of truncated *cyclin D1* transcripts supports this hypothesis (Sander et al., 2005).

Since we observed that the expression of cyclin D1 increased significantly upon truncation of the 3'UTR, it is possible that the varied effects of *cyclin D1* overexpression with or without the UTR are simply due to the differences in cyclin D1 protein expression. This increase

in protein expression could result from the increased stability of *cyclin D1* mRNA upon deletion of mRNA destabilizing elements from the 3'UTR. An important regulatory element in 3'UTRs of various mRNAs that affects their stability is the AU-rich element. A number of studies have hypothesized that AREs in the 3'UTR could potentially destabilize the cyclin D1 3'UTR and therefore their loss could contribute to enhanced stability and therefore overexpression of cyclin D1 protein. We found that mutations in the AUUUA sequences which are signature motifs of AREs, from the cyclin D1 3'UTR actually leads to a significant decrease in cyclin D1 protein levels and an almost complete loss of reporter activity suggesting that in contrast to previous hypotheses, AREs have a potentially stabilizing function on the *cyclin D1* mRNA. Recently, several mRNAs have been shown to be stabilized by the binding of ARE binding proteins to the 3'UTR (Fialcowitz-White et al., 2007; Zhang et al., 2008; Zhao et al., 2008). The elucidation of such factors that bind to the cyclin D1 3'UTR could be interesting. However our results show also that 3'UTR mediated repression of cyclin D1 expression is not mediated by ARE elements but by other factors.

MicroRNAs are small non-coding RNAs which can downregulate the expression of several mRNAs by binding to target sites in their 3'UTRs. They have been shown to act either as tumor suppressors by targeting the expression of oncogenes or as oncogenes by downregulating the expression of tumor suppressor genes (Calin and Croce, 2006). The role of these RNAs in cancer is now increasingly being appreciated (Esquela-Kerscher and Slack, 2006). We explored the possibility that microRNAs target the cyclin D1 3'UTR and identified potential microRNA binding sites in this region. Since microRNAs are known to fine tune the expression of their target mRNAs, the deregulation of microRNA mediated regulation could be an essential prerequisite for cellular transformation in cyclin D1 3'deleted cells. The acquisition of somatic mutations and deletions of this region could render these mRNAs immune to the control of microRNAs. A recently described example is the HMGA2 gene which is frequently mutated in several cancers. This gene was recently shown to harbour seven binding sites for microRNA of the let-7 family. In some tumors, the 3'UTR of this gene is deleted, thereby leading to HMGA2 mRNA transcripts that are immune to regulation by let-7 and consequently, pathological overexpression of the HMGA2 gene (Mayr et al., 2007).

In our study, microRNAs of the miR-15/16 family and the miR-17/92 cluster were shown to directly target the *cyclin D1* 3'UTR and reduce the levels of cyclin D1 protein expression. The genomic amplification of the miR-17-92 cluster has been reported in MCL (Rinaldi et al., 2007). Accordingly, the loss of the binding site of this miRNA from the cyclin D1 gene would promote the constitutive over-expression of this proto-oncogene.

MicroRNAs of the miR-15/16 family have also been implicated in CLLs (Calin et al., 2002) and in recent studies, they have been shown to downregulate several transcripts which regulate cell cycle progression (Linsley et al., 2007). Interestingly we observe that a number of G1 cyclins including CCND2, CCND3, CCNE1 harbour predicted miR-15/16 binding sites. Therefore, it is conceivable that the rapid and efficient turnover of these cyclins, which is a prerequisite for cell cycle progression, could be co-ordinately regulated by these microRNAs. Our demonstration of microRNA targeting of the *cyclin D1* transcripts confirms recent reports showing microRNA mediated regulation of cyclin D1 in MCL and breast cancer (Chen et al., 2008; Yu et al., 2008). Importantly, our data also show that these microRNAs can regulate endogenous cyclin D1 levels in breast cancer cells with an intact 3'UTR but not in 3'UTR deleted cells. This study highlights the important contribution of the cyclin D1 3'UTR in its regulation, the deletion of which could be an important prerequisite for malignant transformation in MCL and solid tumors.

In cancer, there is growing evidence pointing to the role of genomic deletions or premature truncations of the 3'UTR regions of proto-oncogenes (Mayr et al., 2007) which could eliminate microRNA binding sites in the target mRNA, thereby escaping microRNA mediated repression. This mechanism presents an additional and hitherto underappreciated regulation of proto-oncogenes in cancer.

Summary

Deregulation of the cell cycle regulator cyclin D1 in a wide variety of tumors has highlighted the role of cell cycle alterations in cancer. Genomic amplifications, mutations or balanced chromosomal translocations involving this gene are believed to lead to its aberrant overexpression in tumors. Somatic mutations in the 3'UTR of cyclin D1 gene have been reported in breast cancer, neuroblastoma and mantle cell lymphoma patients although their contribution to the cyclin D1 deregulation is unclear. In our study, we confirmed a regulatory role of the 3'UTR in cyclin D1 expression. Our results demonstrated that deletion of the cyclin D1 3'UTR significantly alters cyclin D1 protein expression and function. Similarly, the introduction of mutations observed in MCL patients in the cyclin D1 3'UTR significantly increased the expression of the cyclin D1 protein. These results underline that in malignancies such as MCL, truncation of the 3'UTR due to genomic deletions or somatic mutations is a likely cause of cyclin D1 overexpression.

In order to ascertain whether the deletion of the cyclin D1 3'UTR could impart proliferative properties to cells, thereby contributing to transformation, we assessed the phenotype of fibroblasts retrovirally transduced with cyclin D1 with or without the 3'UTR. Interestingly our results demonstrated marked changes in cyclin D1 function upon deletion of the cyclin D1 3'UTR. Cells expressing cyclin D1 without the 3'UTR proliferated significantly more than those expressing the full length cyclin D1. Similar results were observed in rat ileum epithelial cells which lack the endogenous cyclin D1. Thus our data confirm that the deletion of the 3'UTR confers a proliferative advantage to cells.

Furthermore, in this dissertation, we focused on the different potential regulatory elements of the cyclin D1 3'UTR to assess their role in controlling cyclin D1 expression. We reasoned that elements in the 3'UTR that are responsible for the controlled expression of the cyclin D1 protein are lost in 3'UTR deleted tumors. Therefore, it would be interesting to specifically pinpoint the role of these elements and highlight their contribution to cyclin D1 protein expression. It is assumed that since AU-rich elements (AREs) in the 3'UTR of cyclin D1 could have a potential

destabilizing effect on the cyclin D1 mRNA, their loss could contribute to the observed overexpression of cyclin D1. Importantly, using highly sensitive reporter assays, we showed that the targeted loss of AREs from an otherwise intact 3'UTR leads to a decrease in reporter expression. These results demonstrate that the loss of these cis-acting elements in 3'UTR deleted tumors cannot account for cyclin D1 overexpression and there must be additional factors involved. Using bioinformatic analysis, we identified putative binding sites for microRNAs, small regulatory non-coding RNAs that have been shown to have important roles in cancer. Our study confirmed that microRNAs of the miR-15/16 family and the miR-17-92 cluster directly target the cyclin D1 gene through post-transcriptional regulation. These microRNAs have been shown to be involved in a cell cycle regulation and in a number of malignancies, especially in B-cell lymphoma. The various forms of cyclin D1 generated by deletions or mutations in the 3"UTR of cyclin D1 in tumors exclude these microRNA binding sites.

Taken together, our results demonstrate a regulatory role for the 3'UTR in cyclin D1 expression and function. We show that the deletion of the cyclin D1 3'UTR leads to cyclin D1 overexpression and confers a proliferative advantage to cells. Finally, our results characterize the regulators functions of the different cis and trans-acting elements of the cyclin D1 3'UTR and identify this region as a bona fide target of cell cycle regulatory microRNAs. Extending these findings to other oncogenes, it is conceivable that the escape of 3'UTR mediated regulation by the acquisition of additional mutations of this region is an under-appreciated mechanism in the pathogenesis of cancer.

Zusammenfassung

Die Deregulierung des Zellzyklus-Regulators Cyclin D1 in einer Vielzahl verschiedener Tumorerkrankungen unterstreicht die Bedeutung von Zellzyklus-Veränderungen in Krebserkrankungen. Es wird angenommen, dass genomische Amplifikationen, Mutationen oder auch balancierte chromosomale Translokationen dieses Gens zu seiner aberranten Überexpression in den Tumoren führen. Daneben wurde auch somatische Mutationen der 3'UTR des Cyclin D1-Gens bei Mamakarzinom-, Neuroblastom- und MCL- Patienten berichtet, deren Beitrag zur Cyclin D1-Deregulierung noch unklar war. In dieser Studie konnten wir die regulatorische Rolle der 3'UTR bei der Cyclin D1-Expression bestätigen. So zeigen unsere Ergebnisse, dass eine Deletion der Cyclin D1-3'UTR die Expression und Funktion dieses Proteins signifikant verändert. Genauso konnten wir zeigen, dass die in MCL-Patienten beobachteten Mutationen in den Cyclin D1-3'UTR die Expression des Cyclin D1-Proteins signifikant steigerte. Diese Ergebnisse unterstreichen, dass in der Erkrankung des MCL eine Verkürzung des 3'UTR durch genomische Deletionen oder somatische Mutationen der Cyclin D1-Überexpression wahrscheinlich zugrunde liegt.

Um festzustellen, ob die Deletion der Cyclin D1-3'UTR den betroffenen Zellen eine proliferative Vorteil verschafft und so zur Transformation beiträgt, untersuchten wir den Phänotyp von retroviral mit Cyclin D1 (mit oder ohne 3'UTR) transduzierten Fibroblasten. Interessanterweise zeigten unsere Ergebnisse deutliche Veränderungen der Cyclin D1-Funktion nach Deletion der Cyclin D1-3'UTR. Zellen, die Cyclin D1 ohne 3'UTR exprimierten, proliferierten signifikant stärker als solche mit der vollen, Cyclin D1-Genexpression. Vergleichbare Resultate wurden mit Ileum-Epithelzellen der Ratte erzielt, die kein endogenes Cyclin D1 besitzen. Daher bestätigen unsere Ergebnisse, dass die Deletion des Cyclin D1-3'UTR Zellen zu einem proliferativen Vorteil der betroffenen Zelle führt.

Darüber hinaus untersuchten wir in dieser Dissertation die verschiedenen potentiellen regulatorischen Elemente der Cyclin D1-3'UTR um deren Rolle in der Regulation der Cyclin D1-

Expression zu beurteilen. Dabei gingen wir von der Hypothese aus, dass die Elemente der 3'UTR, die für die Kontrolle der Cyclin D1-Expression verantwortlich sind, in 3'UTR-deletierten Tumoren verloren gehen. Daher ist es von speziellem Interesse, die Rolle dieser Elemente und ihren Auswirkung auf die Cyclin D1-Proteinexpression zu untersuchen. Es wird angenommen, dass der Verlust von AU-reiche Elemente (AREs) in der 3'UTR einen potentiell destabilisierenden Effekt auf die Cyclin D1 mRNA besitzen und ihr Verlust somit zur Überexpression des Cyclin D1-Proteins führt. Durch den Einsatz hochsensitiver Reporter-Assays konnten wir zeigen, dass der spezifische Verlust der AREs in einem ansonsten intakten 3'UTR zu einer Verringerung der Reporterexpression führt. Diese Resultate unterstreichen, dass der Verlust dieser cis-aktiven Elemente der 3'-UTR in Tumoren mit 3'UTR-Deletionen nicht für die Cyclin D1 Überexpression verantwortlich ist sondern zusätzliche Faktoren beteiligt sein müssen. Durch bioinformatische Analyse identifizierten wir mögliche Bindungsstellen für microRNAs, d.h. kleine regulatorische nicht-kodierende RNAs, die eine bedeutende Rolle in Krebserkrankungen spielen. Unsere Untersuchungen bestätigten, dass microRNAs der miR-15/16-Familie sowie des miR-17-92 –Cluster die Expression des Cyclin D1-Gens durch post-transkriptionelle Regulierung direkt beeinflussen. Es ist bekannt, dass diese microRNAs an der Zellzyklus-Regulierung sowie bei einer Reihe maligner Erkrankungen, insbesondere bei B-Zell-Lymphomen beteiligt sind. Den verschiedenen Cyclin D1-mRNAs mit Deletionen oder Mutationen der 3'UTR in Tumoren entstehen, fehlen diese microRNA-Bindungsstellen.

Zusammengefasst bestätigen unsere Ergebnisse die regulatorische Rolle der 3'UTR bei der Cyclin D1-Expression und –Funktion. Wir konnten zeigen, dass die Deletion der Cyclin D1 3'UTR zu einer Cyclin D1-Überexpression führt und zu einem proliferativen Vorteil der betroffenen Zelle führt. Schließlich charakterisieren unsere Ergebnisse die regulatorischen Eigenschaften der verschiedenen cis- und trans-aktiven Elemente der Cyclin D1 3'UTR und identifizieren diese Region als eine bona fide Zielregion der Zellzyklus-regulatorischen microRNAs. Überträgt man diese Resultate auf andere Onkogene ist es denkbar, dass der Verlust der 3'UTR-vermittelten Gen-Regulation durch zusätzliche Mutationen dieser Region einen unterschätzten Mechanismus in der Pathogenese von Krebserkrankungen darstellt.

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