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# **Molecular signatures for CCN1, p21 and p27 in progressive Mantle Cell lymphoma**

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## Abstract

Mantle cell lymphoma (MCL) is a comparatively rare non-Hodgkin's lymphoma characterised by overexpression of cyclin D1. Many patients present with or progress to advanced stage disease within three years. MCL is considered an incurable disease with median survival between 3-4 years. We have investigated the role(s) of CCN1 (CYR61) and cell cycle regulators in progressive MCL.

We have used the human MCL cell lines REC1<G519<JVM2 as a model for disease aggression. The magnitude of CCN1 expression in human MCL cells is REC1>G519>JVM2 cells by RQ-PCR, depicting a decrease in CCN1 expression with disease progression. Investigation of CCN1 isoform expression by western blotting showed that whilst expression of full-length CCN1 was barely altered in the cell lines, expression of truncated forms (18-20 and 28-30 kDa) decreased with disease progression.

We have then demonstrated that cyclin D1 and cyclin dependent kinase inhibitors (p21<sup>CIP1</sup> and p27<sup>KIP1</sup>) are also involved in disease progression. Cyclin D1 was highly expressed in REC1 cells (OD: 1.0), reduced to one fifth in G519 cells (OD: 0.2) and not detected by western blotting in JVM2 cells. p27<sup>KIP1</sup> followed a similar profile of expression as cyclin D1. Conversely, p21<sup>CIP1</sup> was absent in the REC1 cells and showed increasing expression in G519 and JVM2 cells. Subcellular localization detected p21<sup>CIP1</sup>/ p27<sup>KIP1</sup> primarily within the cytoplasm and absent from the nucleus, consistent with altered roles in treatment resistance.

Dysregulation of the CCN1 truncated forms are associated with MCL progression. In conjunction with reduced expression of cyclin D1 and increased expression of p21, this molecular signature may depict aggressive disease and treatment resistance.

**Key words:** CCN1, Cyclin D1, CYR61, MCL, p21<sup>CIP1</sup>, p27<sup>KIP1</sup>.

**Abbreviations:**

MCL	Mantle cell lymphoma
NHL	Non Hodgkin's Lymphoma
CCND1	Cyclin D1
CDK4 or 6	Cyclin-dependent kinase 4 (CDK4) or cyclin-dependent kinase 6
pRb	Retinoblastoma protein
ATM	Ataxia telangiectasia mutated
CYR61	Cysteine-rich protein 61
AML	Acute myeloid leukaemia
SP	Signal peptide
IGFBP	Insulin like growth factor binding domain
VWC	Von Willebrand type C repeat
TSP-1	Thrombospondin type 1 domain
CT	Cysteine rich carboxyl terminal
HSPGs	Heparan sulfate proteoglycans
BMP	Bone morphogenetic protein
TGF- $\beta$	Transforming growth factor $\beta$
VEGF	Vascular endothelial growth factor
MMPs	Matrix metalloproteinases
MM	Multiple myeloma
OSC	Oesophageal squamous carcinoma
MEK/ERK	Mitogen-activated protein kinase/extracellular-signal-regulated kinase
OAFs	Osteoclast activating factors
OBIs	Osteoblast inhibitors
IL-6	Interleukin-6
p21 <sup>CIP1</sup>	(Cdk Interacting Protein 1)
p27 <sup>KIP1</sup>	(Kinase Inhibitory Protein 1)
p57 <sup>KIP2</sup>	(Kinase Inhibitory Protein 2)
TNBC	Triple negative breast carcinoma
FOXO3a	Forkhead box O3
PCNA	Proliferating cell nuclear antigen

OSCC	Oral squamous cell carcinoma
HCC	Hepatocellular carcinoma
NLS	Nuclear Localisation Signal
DCIS	High-grade ductal carcinoma in situ
NSCLC	Non-small-cell lung cancer

## **Introduction**

Mantle cell lymphoma (MCL) is a distinct subset of B-cell Non Hodgkin's Lymphoma (NHL) characterised by overexpression of cyclin D1 as a result of t(11;14)(q13;q32) chromosomal translocation (Pérez-Galán, Dreyling & Wiestner, 2011). This translocation juxtaposes the cyclin D1 gene on chromosome 11q13 with the immunoglobulin heavy chain gene on 14q32 which leads to cyclin D1 overexpression and the dysregulation of the cell cycle (O'Connor, 2007; Zucca & Bertoni, 2013). The clinical course of this disease is highly variable and ranges from indolent classic morphology to aggressive variants with blastoid or pleomorphic morphology (Royo *et al.*, 2012). MCL is characterised by almost inevitable relapses; many patients present with or progress to advanced stage disease within three years due to increasing resistance to chemotherapy and other agents (Dreyling *et al.*, 2018; Liu, Zhang & Zhong, 2015). MCL is broadly considered an incurable disease with the median survival of patients being 3-4 years.

## **The REC1, G519, JVM2 cell line model for MCL progression**

In this study, we have used three human MCL cell lines REC1, Granta 519 (G519) and JVM2 as a model for MCL disease progression. The 2016 revision of World Health Organisation classification of lymphoid neoplasms describes the variants of MCL in addition to the characteristic Cyclin D1 rearranged (i) 2 types of clinically indolent forms with either Immunoglobulin heavy chain variable region gene (IGHV) unmutated or minimally mutated and usually with expression of SOX11 typically involving lymph nodes or extranodal sites, (ii) acquisition of additional molecular or cytogenetic abnormalities leading to an aggressive blastoid or pleomorphic phenotype and (iii) leukaemic non-nodal MCL develops from IGHV mutated and SOX11 negative B cells with usual bone marrow and / or spleen involvement where abnormalities in TP53 contributes to enhance aggressive nature (Swerdlow *et al.*, 2016). REC1 cells display unmutated IGHV with expression of SOX11 (Beà *et al.*, 2013) consistent with the classic indolent forms of MCL. GRANTA 519 (G 519) cells display blastoid phenotype with SOX11 positivity and amplification of BCL2 gene leading to Bcl2 overexpression enhancing cell survival associated with the aggressive blastoid variant forms (Queirós *et al.*, 2016; Rudolph *et al.*, 2004). JVM2 cells are IGHV unmutated and SOX11 negative, expressing low levels of cyclinD1 with increased expression of cyclin D2, BCL2 positive (Tucker *et al.*, 2006) and is considered a blastoid variant (Camps *et al.*, 2006) consistent with the aggressive blastoid variant, SOX11 negativity overlaps with the

aggressive leukaemic non-nodal MCL forms. In addition, the cell lines show increasing resistance to lenalidomide in the order of REC1<G519<JVM2 (Zhang *et al.*, 2008) with G519 and JVM2 cells also showing increased resistance to Ibrutinib (Balsas *et al.*, 2017). REC1 cells are observed as early stage/ indolent MCL showing sensitivity to conventional therapy in contrast to G519 and JVM2 cell lines which behave consistently with aggressive stages (Nordgren, Hegde & Joshi, 2012; Rauert-Wunderlich *et al.*, 2016).

## **Cyclin D1 and cell cycle progression**

Overexpression of cyclin D1 (CCND1) as a result of t(11;14) chromosomal translocation is the hallmark feature of mantle cell lymphoma (Cassaday *et al.*, 2015). Cyclin D1 plays a central role in the cell cycle regulation by binding to either cyclin-dependent kinase 4 (CDK4) or CDK6. The CCND1-CDK4 or CCND1-CDK6 complex phosphorylates the retinoblastoma protein (pRb) which leads to degradation of CCND1 suppressor effect on cell cycle progression. This process leads to release of the E2F family of transcription factors and then S-phase entry (Cassaday *et al.*, 2015). E2F transcription factor regulates genes that encode DNA replication and cell cycle control (Nevins, 2001). In MCL, CCND1 overexpression leads to hyperphosphorylate pRb and accumulation of E2F which facilitates the G<sub>1</sub>/S transition and uncontrolled cell proliferation (Cassaday *et al.*, 2015). CCND1 is upregulated in almost all MCL patients, however cyclin D1 alone is insufficient to promote MCL. Additional oncogenic aberrations have been implicated in the generation of MCL, including, c-Myc overexpression, lack of the ataxia telangiectasia mutated (ATM) gene or p53 dysregulation (Müller *et al.*, 2013). Many secondary genetic events are involved in MCL lymphomagenesis and include inactivation of the DNA damage response pathways, activation of cell-survival pathways, and suppression of apoptosis. Additional oncogenic aberrations are likely to contribute to the development of MCL involving cell proliferation, survival, and interactions with microenvironment (Jares, Colomer & Campo, 2012; Müller *et al.*, 2013). Mutations within stem cell signalling pathways have been identified that may contribute to the pathogenesis of this disease. For example, Notch1 mutations are found in 12% of MCL and are associated with poor survival, suppression of the Notch pathway in MCL, decreased cell proliferation and increased apoptosis (Kridel *et al.*, 2012). Similarly, deregulation of the Wnt canonical pathway was found in MCL by inactivation of phospho-GSK3B suggesting that the Wnt pathway may also contribute to pathogenesis (Gelebart *et al.*,

2008). Further investigations are required to develop novel, effective therapeutic agents for MCL.

Many studies have indicated that activation or upregulation of cell cycle regulators, p21, p27 and cyclin D1 are induced through CCN1 signalling (Sawai *et al.*, 2007; Tong *et al.*, 2004; Xie *et al.*, 2004), whilst the functional effect or output appears to be cell type specific. In 2014, (Saglam *et al.*) have found that induction of cyclin D1 expression in grade ductal carcinoma in situ (DCIS) can occur through CCN1 signalling leading to cell cycle progression. CCN1 protein promoted cell cycle arrest by increased cell senescence at G0/G1 phase through activation notch-1-p21 pathway and reduced proliferation of human trophoblast cells (Kipkeew *et al.*, 2016). Similarly, CCN1 signalling induced accumulation of p53 and p21 driving cell senescence leading to suppression of lung cancer cell proliferation (Jim Leu *et al.*, 2013).

### **CCN1 (CYR61)**

CCN1, a matricellular protein is involved in stem cell signalling within the haematopoietic microenvironment (McCallum & Irvine, 2009; Wells *et al.*, 2015) and has been associated with a number of haematological malignancies including acute myeloid leukaemia (AML) and multiple myeloma (MM) (Crawford & Irvine, 2016). CCN1, also known as CYR61, belongs to CCN family of proteins that are comprised of a signal peptide (SP), Insulin like growth factor binding domain (IGFBP), Von Willebrand type C repeat (VWC), a hinge region leading to the Thrombospondin type I domain (TSP-1) and cysteine rich carboxyl terminal (CT) (Planque & Perbal, 2003).

CCN1 has been shown to be involved in a diverse array of cellular processes including regulation of cell migration, cell adhesion, proliferation, differentiation, apoptosis and angiogenesis through direct binding to cell surface receptors such as integrins and heparan sulfate proteoglycans (HSPGs) (Jandova *et al.*, 2012; Kireeva *et al.*, 1996). CCN1 is a ligand for integrins and acts through direct binding to integrins (via VWC, TSP-1 and CT domains) and HSPGs in order to enhance specific functions (Leu *et al.*, 2004). Increasing evidence has shown that CCN1 promotes cell adhesion by binding to integrin  $\alpha 6\beta 1$ -HSPG co-receptors in fibroblasts (Todorovic *et al.*, 2005),  $\alpha M\beta 2$  in murine macrophages (Bai, Chen & Lau, 2010)  $\alpha D\beta 2$  in macrophage foam cells (Yakubenko, Yadav & Ugarova, 2006),  $\alpha I\beta 3$  in activated platelets (Jedsadayamata *et al.*, 1999). CCN1 may interact with other growth factors such as

bone morphogenetic proteins (BMP), transforming growth factor  $\beta$  (TGF- $\beta$ ) and vascular endothelial growth factor (VEGF) which need more investigation (Lau, 2011). Recently, it has been seen that ALK5 suppression prevents TGF- $\beta$ -induced CCN1 expression in human dermal fibroblasts (Thompson, Murphy-Marshman & Leask, 2014). More importantly, CCN1 is a transcriptional target of TGF- $\beta$  and may potentiate an autocrine regulatory mechanism in tumorigenesis (Bartholin *et al.*, 2007) and is similarly a target of the Wnt/B-catenin pathway as demonstrated in mesenchymal stem cell differentiation to osteoblasts and hepatocellular carcinoma (Si *et al.*, 2006; Li *et al.*, 2012). Constitutive activation of the Wnt Signalling pathway was also identified in MCL (Gelebart *et al.*, 2008), with MCL initiating cells (MCL-ICs) displaying activation of the Wnt signalling pathway (Samaniego *et al.*, 2014). Targeting of Wnt signalling specifically reduces the growth of MCL-ICs (Mathur *et al.*, 2015).

CCN1 can be observed in cancer as “a double-edged sword” (Lau, 2011) where altered CCN1 expression in various cancers may induce or suppress tumour growth (Feng, Wang & Ren, 2008; Holloway *et al.*, 2005). CCN1 plays unique roles in different cancers: it is a tumour promoter in cancers of the breast (Menendez *et al.*, 2005), prostate (Sun *et al.*, 2008), pancreatic (Maity *et al.*, 2014), gastric carcinogenesis (Cheng *et al.*, 2014), ovarian carcinoma (Gery *et al.*, 2005), colorectal (Jeong *et al.*, 2014), myeloma (Roodman, 2014) and acute myeloid leukaemia (AML) (Niu *et al.*, 2014) due to either overexpression of CCN1 or truncated isoforms. Paradoxically, CCN1 is a tumour suppressor in melanoma (Dobroff *et al.*, 2009), non-small cell lung cancer (Tong *et al.*, 2001) and endometrial adenocarcinoma (Chien *et al.*, 2004). Full-length CCN proteins can play an anti-proliferative role, while truncated isoforms may induce tumour proliferation (Planque & Perbal, 2003). For example, when CCN1 is cleaved by plasmin, this releases a truncated isoform of CCN1 (28kDa) supporting endothelial cell migration in breast carcinoma cells (Pendurthi *et al.*, 2005). In 2013, (Choi *et al.*, 2013) identified truncated CCN1 in the vitreous fluid secretome of proliferative diabetic retinopathy patients rather than full-length protein. Furthermore, a truncated isoform of CCN1 which includes complete or partial length forms of the two-modules form IGFBP-VWC is generated by proteolytic cleavage of matrix metalloproteinase MMPs (Choi *et al.*, 2013). In addition to proteolysis generating production of a CCN1 truncated isoform, it may also be generated by alternative mRNA splicing (Perbal, 2009).

Increasing evidence suggests that CCN1 plays important roles in tumour development, including migration, survival, proliferation and metastasis (Sun *et al.*, 2008). In AML, CCN1 induces tumour survival through activation of the Ras/Raf/MEK/ERK pathway by up

regulating c-Myc and Bcl-xL and by down regulating Bax (Niu *et al.*, 2014). Similarly, CCN1 confers human breast cancer resistance to chemotherapeutic agent-induced apoptosis and suppresses apoptosis by activating the integrins/NF-kb/XIAP signalling pathway (Lin *et al.*, 2004). CCN1 has been found in sites of bone remodelling, involvement enhancing osteoblast differentiation while suppressing osteoclast formation (Si *et al.*, 2006).

Furthermore, CCN1 has been implicated in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells (Si *et al.*, 2006). *In vivo*, (Johnson *et al.*, 2014) have reported that overexpression of CCN1 which produced by mesenchymal cells in bone marrow microenvironment of patients with monoclonal gammopathy of undetermined significance (MGUS), asymptomatic myeloma (AMM), and multiple myeloma (MM) suppressed tumour cell growth and reduced MGUS progression to MM. However, CCN1 may enhance myeloma cell viability through supporting survival of the INA-6 myeloma cell line lacking interleukin-6(IL-6) (Dotterweich *et al.*, 2014).

Many studies have indicated that activation or upregulation of cell cycle regulators, p21, p27 and cyclin D1 induce through CCN1 signalling (Sawai *et al.*, 2007; Tong *et al.*, 2004; Xie *et al.*, 2004). In 2014, (Saglam *et al.*) have found that induction of cyclin D1 expression in grade ductal carcinoma in situ (DCIS) can occur through CCN1 signalling leading to cell cycle progression. However, CCN1 protein promoted cell cycle arrest by increased senescence-cell at G0/G1 phase through activation notch-1-p21 pathway and reduced cell proliferation of human trophoblast cells (Kipkeew *et al.*, 2016). Moreover, CCN1 signalling induced accumulation of p53 and p21 driving senescence of cell leading to suppress lung cancer cell proliferation (Jim Leu *et al.*, 2013).

## **p21**

p21<sup>CIP1</sup> (p21) belongs to the CIP/KIP family of proteins that regulate cell cycle progression. The CIP/KIP family comprises three members; p21<sup>CIP1</sup> (Cdk Interacting Protein 1), p27<sup>KIP1</sup> (Kinase Inhibitory Protein 1) and p57<sup>KIP2</sup> (Kinase Inhibitory Protein 2) (Bretones, Delgado & León, 2015) which bind and inhibit most cyclin-CDK complexes. The CIP/KIP family of proteins, particularly p21, plays an essential role in cell cycle control, halting the transition from G<sub>1</sub> phase to S phase (Pérez-Sayáns *et al.*, 2013). Two pathways regulate p21; a p53-dependent pathway (in response to DNA damage which activates p53 leading to upregulation of p21 and repression of cell growth in G<sub>1</sub> phase with potential DNA repair or stimulation of programmed cell death) and a p53-independent pathway, in which cellular growth factors

regulate p21 expression (Brennan *et al.*, 2002; Ciccarelli *et al.*, 2005). p21 binds to CCND1-CDK4 or -CDK6 complex and inhibits the kinase activity of CDKs in response to many stimuli leading to regulation G<sub>1</sub>/S progression, at the restriction point. This process leads to repression of phosphorylation of pRb protein which in turn prevents the expression of E2F factors and blocks G<sub>1</sub>/S transition (Zhang & Yan, 2012).

In addition to negative regulation of the cell cycle, p21 can regulate gene transcription; p21 suppresses E2F transcription factor through a pathway independent of CDKs or Rb (Perkins, 2002). p21 is involved in controlling cellular growth by suppressing of E2F1 transcription factor via Wnt4 expression and Notch 1 activation (Devgan *et al.*, 2005) and p21 stimulates NF $\kappa$ b-mediated transcription by activation of p300 and CBP (Perkins, 2002). Studies have identified additional roles for p21, in cancer it is a tumour suppressor through cell cycle arrest and blocking DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) (Waga *et al.*, 1994) or as oncogenic factor, promoting carcinogenesis and tumour development (Roninson, 2002). The expression of p21 varies in different cancers, it is down regulated in small-cell lung (Komiya *et al.*, 1997), colorectal (Zirbes *et al.*, 2000), cervical (Lu *et al.*, 1998a), and head and neck cancer (Kapranos *et al.*, 2000) that are associated with tumour progression. In contrast, it is upregulated in prostate (Baretton *et al.*, 1999), ovarian (Ferrandina *et al.*, 2000), breast, oesophageal squamous cell carcinomas, and in brain tumours (Roninson, 2002). The function of p21 depends on subcellular localization which may be nuclear, cytoplasmic or mitochondrial. p21 regulates cell proliferation and differentiation by localizing in the nucleus (Abbas & Dutta, 2009), whilst p21 enables resistance to DNA damage by inhibiting proteins essential for apoptosis by localising to the cytoplasm or mitochondria, enhancing tumourigenesis (de Renty, DePamphilis & Ullah, 2014; Sohn *et al.*, 2006)

## **p27**

In G<sub>1</sub> phase of cell cycle, reviewed in (Hirama & Koeffler, 1995) p27 binds to CCNE-CDK2 complex and inhibits the catalytic activity of CDK2 resulting in cell cycle arrest at the restriction point in response to DNA damage or anti-mitogenic signals. As a result, this prevents the phosphorylation of pRb which leads to block in the transcription of genes required for G<sub>1</sub>/S progression (Toyoshima & Hunter, 1994). Beyond the restriction point, cell cycle proceeds independent of mitogenic signals (Coats *et al.*, 1996).

Interestingly, p27<sup>KIP1</sup> and p21<sup>CIP1</sup> have an important role in promotion of assembly of CCND-CDK4/6 complexes (LaBaer *et al.*, 1997). This interaction leads to sequestration of p27 in CCND-CDK4 complex which blocks inhibition of the CCNE-CDK2 complex (Perez-Roger *et al.*, 1999). Binding of p27<sup>KIP1</sup> with CCND-CDK4 complex suppresses the kinase activity of CDK4 (Ray *et al.*, 2009). Expression of p27<sup>KIP1</sup> is reduced in several types of cancer associated with poor prognosis including breast (He *et al.*, 2012), prostate (Roy *et al.*, 2008), lung and colon cancer (Timmerbeul *et al.*, 2006). In contrast, p27<sup>KIP1</sup> is overexpressed in hepatocellular carcinoma (HCC) which is associated with longer disease free survival (Qin & Ng, 2001). In MCL, Quintanilla-Martinez *et al.* (2003) suggested overexpression of cyclin D1 contributed to a change in p27<sup>KIP1</sup> levels leading to inhibition of cellular growth.

In addition to cell cycle regulation, there is some evidence that p27<sup>KIP1</sup> has important roles in apoptosis, transcriptional activation, and migration depending on its localization. In the nucleus, it has an essential role to inhibit cell growth and is considered as a tumour suppressor (Jeannot *et al.*, 2015). Phosphorylation of specific sites on p27<sup>KIP1</sup> leads to its export into the cytoplasm where it can act as a tumour promotor as reviewed in (Besson, Dowdy & Roberts, 2008). Many studies have shown that cytoplasmic p27<sup>KIP1</sup> in cancer including melanoma (Chen *et al.*, 2011; Denicourt *et al.*, 2007), ovarian carcinoma (Duncan *et al.*, 2010), renal cell carcinoma (Kruck *et al.*, 2012), osteosarcoma (Li *et al.*, 2016) and acute myelogenous leukaemia (Min *et al.*, 2004) is associated with cell migration, high tumour grade and metastasis, poor prognosis and survival. Moreover, cytoplasmic p27<sup>KIP1</sup> contributed to treatment resistance mediated suppression of apoptosis in Her2+ breast cancer cells (Zhao *et al.*, 2014).

This study investigated the expression of CCN1 and cell cycle regulators Cyclin D1, p21 and p27 in progressive MCL.

## **Materials and Methods**

### **Cell culture**

Human Mantle Cell Lymphoma cell lines REC1, G519 and JVM2 were purchased from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ-Germany). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% FBS (Gibco) and were incubated at 37° C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were passaged twice weekly to maintain log phase. Experiments were conducted within 10 passages from cell recovery and cells were seeded at 2x10<sup>5</sup> cells per ml for experimental procedures. Cells were counted using a haemocytometer and viability assessed using the trypan blue exclusion assay.

Normal peripheral blood (nPB) samples were obtained from Normal blood donors (n=5) via NHS Blood and Transplant Bristol (NHSBT, Bristol, UK) with material transfer agreement and approval for use via Plymouth University ethics committee. Lymphocytes were extracted using Lymphoprep™ (Axis Shield, UK) using manufacturer's instructions and cells harvested in Trizol® reagent (Fisher Scientific, UK) for RNA analyses.

### **Protein extraction and quantification**

Total cell lysates for three cell lines (REC1, G519 and JVM2) were prepared in Radioimmune Precipitation Assay buffer (RIPA): Tris 50 mM, NaCl 150 mM, Triton X-100 1%, Na-Deoxycholate 0.5%, SDS 0.1% supplemented with Complete™ protease inhibitor (Roche UK) and stored at -20 °C until required.

### **Subcellular Fractions**

Nuclear, mitochondria and cytoplasmic proteins from REC1, G519 and JVM2 cell lines were extracted using the Cell Fraction Kit-Standard (Abcam, UK) according to manufacturer instructions with the following modifications: reduced cell number to 1x10<sup>6</sup>/ml incubated with 500 µl of buffer A (1X) and samples were incubated for 8 minutes to extract clean mitochondrial fractions. Buffer A (1X) was prepared using 5 ml 2X buffer A stock, 4900 µl dH<sub>2</sub>O and 100µl PI 100X (Halt Protease Inhibitor Single-Use Cocktail EDTA-Free, ThermoScientific). All fractions were stored at -80 °C until required.

Protein was quantified using the micro BCA™ Assay Kit following the supplier instructions (Pierce, ThermoScientific, UK). The subcellular fractions for nuclear, cytoplasmic and mitochondrial extracts were run simultaneously on one gel (NuPage 10 well 10% Bis Tris gel (Invitrogen, UK)) to enable direct comparison. Experiments were conducted in triplicate (n=3).

## **Western blotting**

Protein samples (10 µg) were loaded on a 10% NuPAGE® Bis-Tris Gels (Invitrogen UK) and transferred onto a PVDF membrane (Millipore, UK). Membranes were blocked with 2.5% skimmed milk for 30 minutes. Membranes were then incubated overnight at 4 °C with primary antibody (rabbit anti-p21<sup>CIP1</sup> (2947S) (1:1000, Cell Signalling Technology), rabbit anti-p27<sup>KIP1</sup> (3688s) (1:1000, New England Biolabs), mouse cyclin D1 (1:1000, Cell Signalling Technology), rabbit anti-CCN1 (ab24448, Lot # GR26258-7) (1:2000, Abcam), anti-GAPDH (ab9485) (1:2500, Abcam), mouse anti-COX IV (ab33985) (1:1000, Abcam), rabbit anti Histone H3 (9717s)(1:1000, New England Biolabs)). Membrane was washed with PBS-T and incubated with an appropriate secondary antibody conjugated to HRP. Bands were detected using chemiluminescence (Thermo Scientific SuperSignal®West Dura Extended Duration Substrate) and viewed using the Image Quant LAS4000.

## **RNA extraction and cDNA synthesis**

Total RNA was extracted using Trizol® reagent (Fisher Scientific, UK). Trizol (1ml for 1x10<sup>6</sup> cells) was pipetted up and down several times until the samples were homogenised. 200 µl of chloroform was added per ml of Trizol®, shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 13000 rpm for 15 minutes at 4 °C. The upper layer was decanted to new tubes and 500 µl isopropanol added and incubated at room temperature for 10 minutes. Samples were centrifuged at 13000 rpm for 15 minutes at 4 °C. Supernatant was discarded and the white RNA pellet washed three times with 70% ethanol. RNase free water was used to re-suspend the pellet. Samples were stored at -80 °C until required. RNA quantity and purity was measured using a nanodrop spectrophotometer using A260/A280 ratios (Thermo-Fisher scientific, Waltham, MA, USA). cDNA was synthesised using 2 µg of total RNA in 20 µl volume using the High Capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer's instructions. cDNA

samples were stored at -20 °C until required. Negative template controls consisting of reaction without cDNA were run for all experiments.

## **RQ-PCR**

The quantitative real-time PCR was performed by using StepOne Software v2.3 analytical software (Thermo-cycler 96 well plate Real time PCR, Applied Biosystems, US) in a volume of 12.5 µl containing 1µl cDNA (100ng), 0.625 µl ppp, 6.25 µl 2xMM (Prime time Master Mix, Integrated DNA technologies). The PCR reaction conditions were 95 °C for 3 minutes, followed by 40 cycles comprising denaturation at 95 °C for 5 seconds, annealing at 60 °C for 30 seconds. Predesigned assay reagents with FAM/ TAMRA fluorescence were used for CCN1 (Hs00155479\_m1), GAPDH ((PT.39a.22214836) from Integrated DNA Technologies), p21 (00355782-M1), p27 (01597588-M1) and cyclin D1 ((00765553-M1) from Applied Biosystems). GAPDH used as an endogenous control, gene expression levels were reported using the  $\Delta\Delta CT$  method with experimental samples run in triplicate and independent replicates of RNA completed in triplicate for reporting.

## **Statistics**

Statistical analysis was performed on data from at least 3 independent experiments. For RQ-PCR, samples were run in triplicate and then independent replicates performed in triplicate for publication. Students t-test was performed to identify significance between samples and where  $p < 0.05$  was deemed significant.

## Results

### CCN1 expression is inversely correlated with MCL aggressiveness

In order to assess the potential role(s) of CCN1 in MCL, we have investigated CCN1 expression using RQ-PCR and Western blotting. REC1, G519, JVM2 human MCL cell lines were used as a model for MCL disease progression in the order from indolent to aggressive disease REC1<G519<JVM2 respectively.

RQ-PCR for CCN1 expression shows an inverse relationship with disease aggression. Normal peripheral blood (nPB) lymphocytes from donors were also assessed for CCN1 expression. CCN1 was barely detected in nPB (CT=34.9 ± 0.75) and therefore set to a fold change of 1 to assess relative expression within the MCL cell lines. CCN1 expression was high in REC1 cells (Fold change 67.18 ± 0.14) and sequentially decreased in progressive G519 cells (Fold change 20.1 ± 0.22) and JVM2 cells (Fold change 2.86 ± 0.52) (Figure 1A).

Investigation of CCN1 total protein expression using western blot analysis showed CCN1 protein expression at the following approximate molecular weights; 42kDa consistent with expression of full length CCN1, 28-30kDa and 18-20kDa consistent with expression of truncated proteins. Expression of full-length CCN1 barely altered through the cell lines however, expression of the truncated form (18-20kDa) was high in REC1 cells (OD:1.0) reduced in G519 cells (OD:0.5) and barely detected in JVM2 cells (Figure 1B and Fig 1C). Reports from a previous study (Choi *et al.*, 2013) suggests the 28-30kDa moiety could comprise the SP, IGFBP, VWC and TSP-1 domains whilst the 18-20 kDa moiety could be either the SP, IGFBP, VWC fragment or TSP-1 and CT fragment, or potentially a mix of both (Figure 1D).

### Cyclin D1 is downregulated in progressive MCL

We have investigated cyclin D1 expression using RQ-PCR and Western blotting using total protein extracts from the three MCL cell lines; REC1, G519 and JVM2. RQ-PCR shows cyclin D1 expression is high in REC1 cells and decreased in G519 and JVM2 cells consistent with deregulation of cyclin D1 in aggressive disease (Figure 2A). Fold changes in cyclin D1 expression were 10.1, 4.6 and 1.0 for REC1, G519 and JVM2 respectively.

Cyclin D1 total protein expression mirrored that of the gene expression where cyclin D1 was highly expressed in the Rec1 cells (OD: 1.0), reduced to one fifth in the G519 cells (OD: 0.2) and not detected by western blotting in the JVM2 cell line (Figure 2B and 2C).

### **High expression of p21<sup>CIP1</sup> and down expression of p27<sup>KIP1</sup> in aggressive MCL**

Cyclin dependent kinase inhibitors, p21<sup>CIP1</sup> and p27<sup>KIP1</sup>, were also investigated for an involvement in disease progression.

RQ-PCR shows increasing expression of p21 with disease progression whilst expression levels of p27 are decreased with disease progression in G519 and JVM2 cells (Figure 2D). Total cell lysates showed that p21<sup>CIP1</sup> was not detected in REC1 cells but had increasing expression in G519 (OD: 0.6) and JVM2 cells (OD: 1.0) (Figure 2E and F). Whilst RQ-PCR and western blotting show that p27<sup>KIP1</sup> expression was high in the REC1 and decreased with disease progression in G519 and JVM2 cells (Figures 2G-I). Total cell lysates showed that p27<sup>KIP1</sup> was high expressed in REC1 (OD: 1.5) and decreased in G519 (OD: 1.0) and not detected in JVM2 cells (Figures 2H and 2I).

### **Altered subcellular localization of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> portray resistance in progressive MCL**

To investigate the subcellular localization of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> in MCL, we extracted cytoplasmic, mitochondrial and nuclear protein from cells and performed western blotting.

Expression of p21<sup>CIP1</sup> was not detected in any fraction for early stage REC1 cells. For the progressive G519 and JVM2 cells, p21<sup>CIP1</sup> was primarily expressed in the cytoplasm and was not detected in the nucleus (Figure 3A). Expression of p21<sup>CIP1</sup> was detected in the mitochondrial fraction for JVM2 cells. Blotting for GAPDH (cytoplasmic), COX IV (Mitochondrial) and Histone 3 (Nuclear) markers were used as controls for each fraction.

For p27<sup>KIP1</sup>, expression was only detected in the cytoplasmic fraction and not in mitochondrial or nuclear fractions (figure 3B). GAPDH (cytoplasmic), COX IV (Mitochondrial) and Histone 3 (Nuclear) markers were used as loading controls and to ensure clean fractions were obtained for each, without cross contamination during the fractionation process.



## Discussion

MCL is considered an aggressive disease and has one of the worst outcomes among B-cell lymphomas due to the dysregulation of the DNA damage response pathways accompanied with abnormal cell survival mechanisms suppressing apoptosis (Campo & Rule, 2015; Moros *et al.*, 2014). MCL characterised by overexpression of cyclin D1 as a result of t(11;14) chromosomal translocation that leads to dysregulation of the cell cycle (O'Connor, 2007; Zucca & Bertonni, 2013). Furthermore, MCL is characterised by clinical course where variants are subdivided into indolent form (classic morphology) and aggressive (blastoid or pleomorphic appearance) (Kridel *et al.*, 2012). The heterogeneous biology and aggressive behaviour of MCL present a challenge for designing standard therapies (Smith, 2011) and therefore require further investigation to identify more effective treatment strategies.

In this study, we have investigated the role(s) of CCN1 in MCL and investigated cell cycle regulation using expression of cyclin D1, p21<sup>CIP1</sup> and p27<sup>KIP1</sup> in the cell line model representing disease progression in MCL where the magnitude of aggressive behaviour is in the order REC1<G519<JVM2. CCN1 dysregulation was identified in MCL progression where CCN1 was highly expressed in the REC1 cells and reduced in the aggressive G519 and JVM2 cells. CCN1 expression decreases with disease progression. We found that the lower expression of CCN1 in aggressive MCL cell lines (G519 and JVM2) is additional risk factor for disease progression. However, many studies have indicated that the high expression of CCN1 is implicated in disease progression, tumorigenesis and invasion of hepatocellular carcinoma (HCC) (Li *et al.*, 2012), breast cancer (O'Kelly *et al.*, 2008), prostatic carcinoma (D'Antonio *et al.*, 2010), gliomas (Xie *et al.*, 2004b) and gastric cancer (Lin *et al.*, 2007).

Further investigation demonstrated that whilst full-length CCN1 remains relatively constant within the cell lines, the 18-20 kDa truncated form is decreased within aggressive G519 and JVM2 cells. Previous reports identify that this truncated form could potentially be a fragment consisting of the SP-IGFBP-VWC domains or the TSP-1-CT domains (Choi *et al.*, 2013) inferring that either transcriptional control or post-translational control of CCN1 (via degradation by MMP2 / MMP14 for example (Choi *et al.*, 2013)) is lost within aggressive MCL. In 2006, Planque *et al.*, have found that full length of CCN3 inhibited cell growth whilst a truncated isoform induced “morphological transformation of chicken embryo fibroblasts” suggesting a role in oncogenic activity. The truncated isoform of CCN3 translocated to the nucleus of cancer cell lines supporting the role of the carboxyl terminal

being involved in transcriptional regulation. Increasing evidence indicates the Nuclear Localisation Signal (NLS), a short peptide motif mediating nuclear localisation of proteins, is located in the CT module of CCN3 (lysine-rich **PTDKKGKKCLRTKKS**SLKA) (Planque, 2006). Perbal (1999) found nuclear localisation of the truncated isoform of CCN3 (NOV) protein (31/32kDa) in the nucleus of 143 and HeLa cells. It is suggested the truncated isoform may be deficient of N-terminus to have a role in the gene expression of target cells since antibodies against the C-terminus of CCN3 / NOV were used (**KKGKKCLRTKKS**). CCN1 (CYR61) and CCN3 (NOV) are members in the CCN family of matricellular proteins sharing 40%-60% amino acid homology which may infer potential for the CCN1 truncated isoform to have nuclear localisation and role(s) in gene regulation (Perbal, 1999; Planque & Perbal, 2003). Supporting this, the CCN3 NLS region (**PTDKKGKKCLRTKKS**SLKA)(Planque, 2006) is highly conserved in the CCN1 CT domain (**KKGKKCSKTKKS**). This may suggest that the CCN1 truncated form (18-20 k Da) may translocate to the nucleus of MCL cell lines. This is consistent with CCN1 found in the nucleus of bladder smooth muscle cells (Chen & Du, 2007; Tamura *et al.*, 2001).

Full length CCN proteins can play an anti-proliferative role, whilst truncated isoforms may induce tumour proliferation (Planque & Perbal, 2003). The truncated isoform appears to have altered biological function(s) owing to CCN1 partition into the soluble phase (28kDa), diffusing freely within tissue and may act as an antagonist towards the full-length CCN1 form within the insoluble matrix (42kDa) (Pendurthi *et al.*, 2005). For example, CCN1 is cleaved by plasmin and releases a truncated form of CCN1 (28kDa) which may support endothelial cell migration in breast carcinoma (Pendurthi *et al.*, 2005). In 2013, (Choi *et al.*), found the CCN1 truncated form (11-23kDa) expressed in diabetic retinopathy patients instead of the full-length 42kDa protein. It is also postulated that a truncated isoform of CCN1 can arise due to alternative mRNA splicing (Perbal, 2009).

In this study, Cyclin D1 was also deregulated in MCL and our findings are consistent with previous reports of cyclin D1 down regulation and disease progression (Peng, Chou & Hsu, 1998). Interestingly, Saglam *et al.* (2014) have demonstrated that the up regulation of cyclin D1 and p53 are activated by the CCN1 pathway in high-grade ductal carcinoma in situ (DCIS). Consistent with these findings, we showed a positive association between CCN1 and cyclin D1 expression in all MCL cell lines. In other solid tumours, where cyclin D1 is overexpressed; breast, liver, lung, and brain cancer (Gillett *et al.*, 1996; Hall & Peters, 1996;

Molenaar *et al.*, 2008) requires consistent signalling from the extracellular matrix and growth factors (Assoian & Klein, 2008).

MCL progression involving down regulation of cyclin D1 and the up regulation of p21<sup>CIP1</sup> may contribute to treatment resistance (Abukhdeir & Park, 2008). We have shown that p21<sup>CIP1</sup> levels increase with disease progression. This is consistent with other studies that report overexpression of p21<sup>CIP1</sup> was correlated with tumour progression; in breast (Ceccarelli *et al.*, 2001) and ovarian carcinoma (Ferrandina *et al.*, 2000) and in brain tumours (Jung *et al.*, 1995).

We have shown that p27<sup>KIP1</sup> levels decrease with disease progression also consistent with findings from Izban *et al.* (2000), where p27<sup>KIP1</sup> was overexpressed in early stage disease (typical MCL) and down regulated in aggressive stage (blastoid variants) where it was associated with a high proliferation rate of blastoid MCL. Conversely, in 1998 (Quintanilla-Martinez *et al.*) showed expression of p27<sup>KIP1</sup> was inversely associated with the proliferation rate of MCL cells; undetected in typical MCL cells (classic disease) associated with low proliferation rate but was overexpressed in the blastic variant of MCL cells (aggressive disease) with higher proliferation rate.

p27<sup>KIP1</sup> and p21<sup>CIP1</sup> have important roles in promotion of assembly of CCND-CDK4/6 complexes (LaBaer *et al.*, 1997). This interaction leads to sequestration of p27<sup>KIP1</sup> in CCND-CDK4 complex which blocks inhibition of the CCNE-CDK2 complex (Perez-Roger *et al.*, 1999). Furthermore, investigation of p21<sup>CIP1</sup> expression in MCL progression showed down regulation at early stage disease and overexpression at advanced stage that mirrors its roles in MCL progression.

More importantly, in cancer, the tumour suppressor function of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> depends on their nuclear localization (Jeannot *et al.*, 2015; Romanov, Pospelov & Pospelova, 2012). Many studies have found that phosphorylation of specific sites of p27<sup>KIP1</sup> and p21<sup>CIP1</sup> lead to their export into the cytoplasm where they can act as a tumour promoters and induce drug resistance (Ohkoshi, Yano & Matsuda, 2015; Zhao *et al.*, 2014). In this study, p21<sup>CIP1</sup> and p27<sup>KIP1</sup> were found in the cytoplasmic fractions and absent in nuclear fractions of all three cell lines (REC1, G519 and JVM2). This suggests in MCL, p21<sup>CIP1</sup> and p27<sup>KIP1</sup> have lost their tumour suppressor roles and acquired tumour promoter roles by localisation to the cytoplasm. Whilst mutation of p21<sup>CIP1</sup> has not been investigated here, mutation of the p21<sup>CIP1</sup> gene frequently occurs in cancer cells leading to inactivation of p21<sup>CIP1</sup> with loss of function

to block the cell cycle, even when overexpressed (Lu *et al.*, 1998b; Lukas *et al.*, 1997). While regulation of p27<sup>KIP1</sup> is different from other cell cycle inhibitors, p27<sup>KIP1</sup> gene mutation is rare (Garrett-Engele *et al.*, 2007).

CCN1 likely plays key role(s) in haematopoiesis and in B cell development through modulation of stem cell signalling pathways, TGF  $\beta$ , BMP, Notch, Wnt- $\beta$  catenin (McCallum & Irvine, 2009; Wells *et al.*, 2015). CCN1 roles within haematological malignancy show CCN1 promotes survival and inhibits apoptosis in AML (Niu *et al.*, 2014) and overexpression of CCN1 in multiple myeloma (MM) postponed tumour growth and suppressed bone destruction (Johnson *et al.*, 2014). CCN1 signalling involves many stem cell pathways active within the bone marrow microenvironment where haematopoiesis ensues; CCN1 can activate the Wnt- $\beta$  catenin-TCF4 signalling pathway in glioma cell (Xie *et al.*, 2004a) and induces Wnt3A osteoblast differentiation of mesenchymal stem cells (Si *et al.*, 2006). CCN1 in some cancers plays important roles in enhancing apoptosis, suppressing tumour growth, such as non-small-cell lung cancer (NSCLC) cell lines through activating the  $\beta$ -catenin-c-myc-p53-p21 signalling pathway (Tong *et al.*, 2004). Moreover, CCN1 enhances pancreatic cancer cell motility *in vitro* and cell tumorigenic growth *in vivo* by regulating sonic-Hedgehog through integrin-Notch-signalling pathway (Haque *et al.*, 2012).

In conclusion, CCN1 expression appears to be regulated in MCL, where reduced expression of the truncated forms (18-20 and 28-30 kDa) is associated with aggressive disease. In combination with reduced expression of cyclin D1 and increased expression of p21, this molecular signature may depict aggressive disease and treatment resistance. Further investigation will ascertain CCN1 role(s) in MCL progression.

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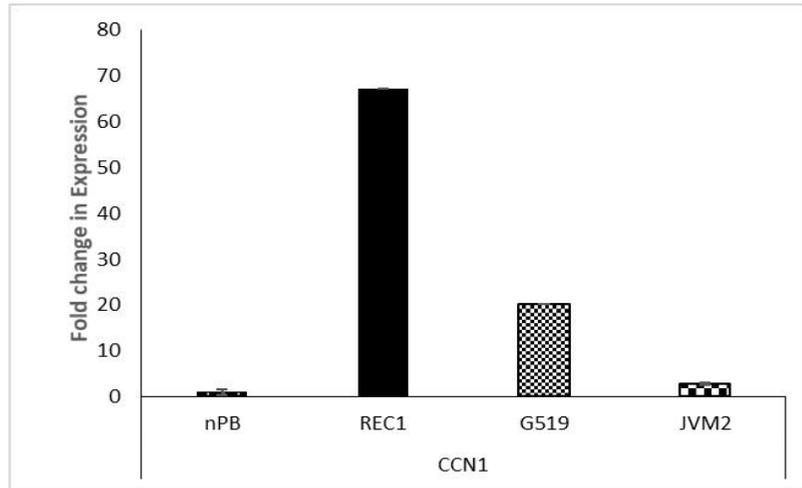
## Legends

**Figure 1 CCN1 expression in MCL progression.** (A) RQ-PCR screen of MCL cell lines for CCN1 expression in normal peripheral blood (nPB), low-aggression phenotype (REC1) and in aggressive disease (G519 and JVM2). (B) Western blot showing CCN1 expression for the MCL cell lines, REC1, G519 and JVM2 (C) Optical densitometry for CCN1 band pattern by Western Blotting. (D) CCN1 domain structure of full length and potential truncated CCN1 proteins adapted from Choi *et al.*, 2013. GAPDH was used as a loading control, data generated were normalised against GAPDH control (n=3 independent samples) and (nPB, n=5 independent samples).

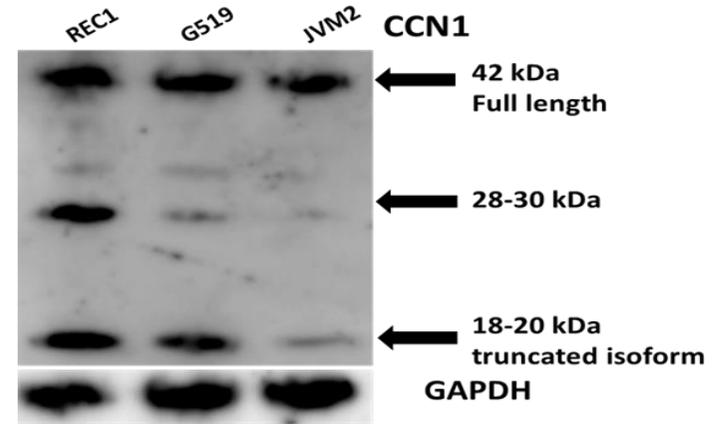
**Figure 2 Cyclin D1, p21 and p27 expression in MCL progression.** RQ-PCR of cyclin D1 (A), p21 (D) and p27 (G) in MCL for REC1, G519 and JVM2 cells. Western blotting for expression of total protein for cyclin D1 (B), p21 (E) and p27 (H) in MCL for REC1, G519 and JVM2 cells. Optical densitometry for western blotting of cyclin D1 (C), p21 (F) and p27 (I). Densitometry was performed on banding and data generated normalised against GAPDH loading control (n=3 independent replicates).

**Figure 3 Subcellular localisation of p21 and p27 in MCL.** A Western blot for p21 cellular content for cytoplasmic, mitochondrial and nuclear fractions for REC1, G519 and JVM2 cells. B Western blot for p27 cellular content for cytoplasmic, mitochondrial and nuclear fractions for REC1, G519 and JVM2 cells. GAPDH, Histone 3 and COXIV were used as a loading control and to ensure clean fractions free from cross contamination during the fractionation process were obtained (n=3 independent samples).

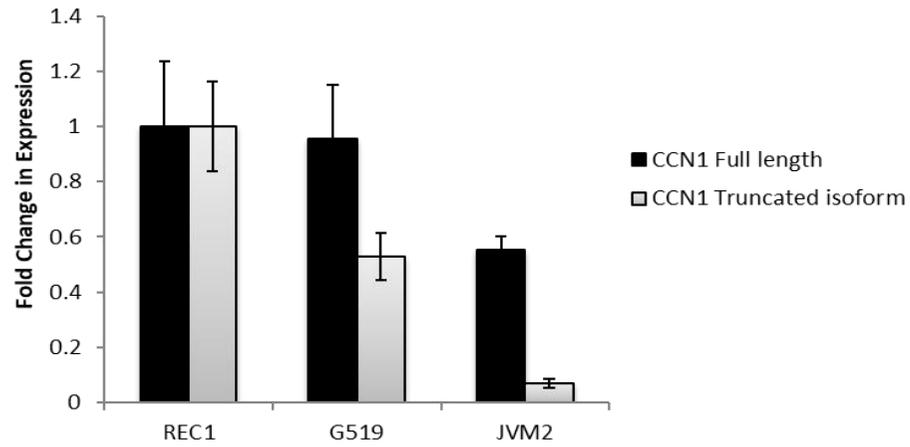
**Figure 1**



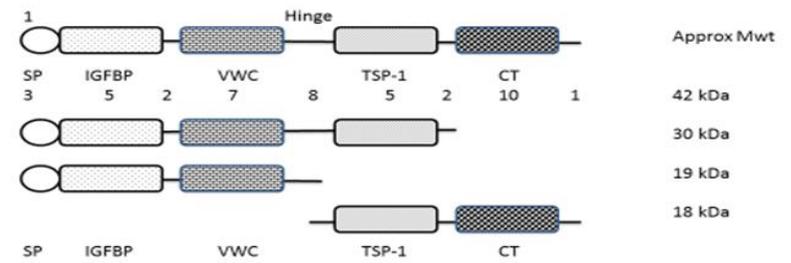
**(A)**



**(B)**



**(C)**



**(D)**

**Figure 2**

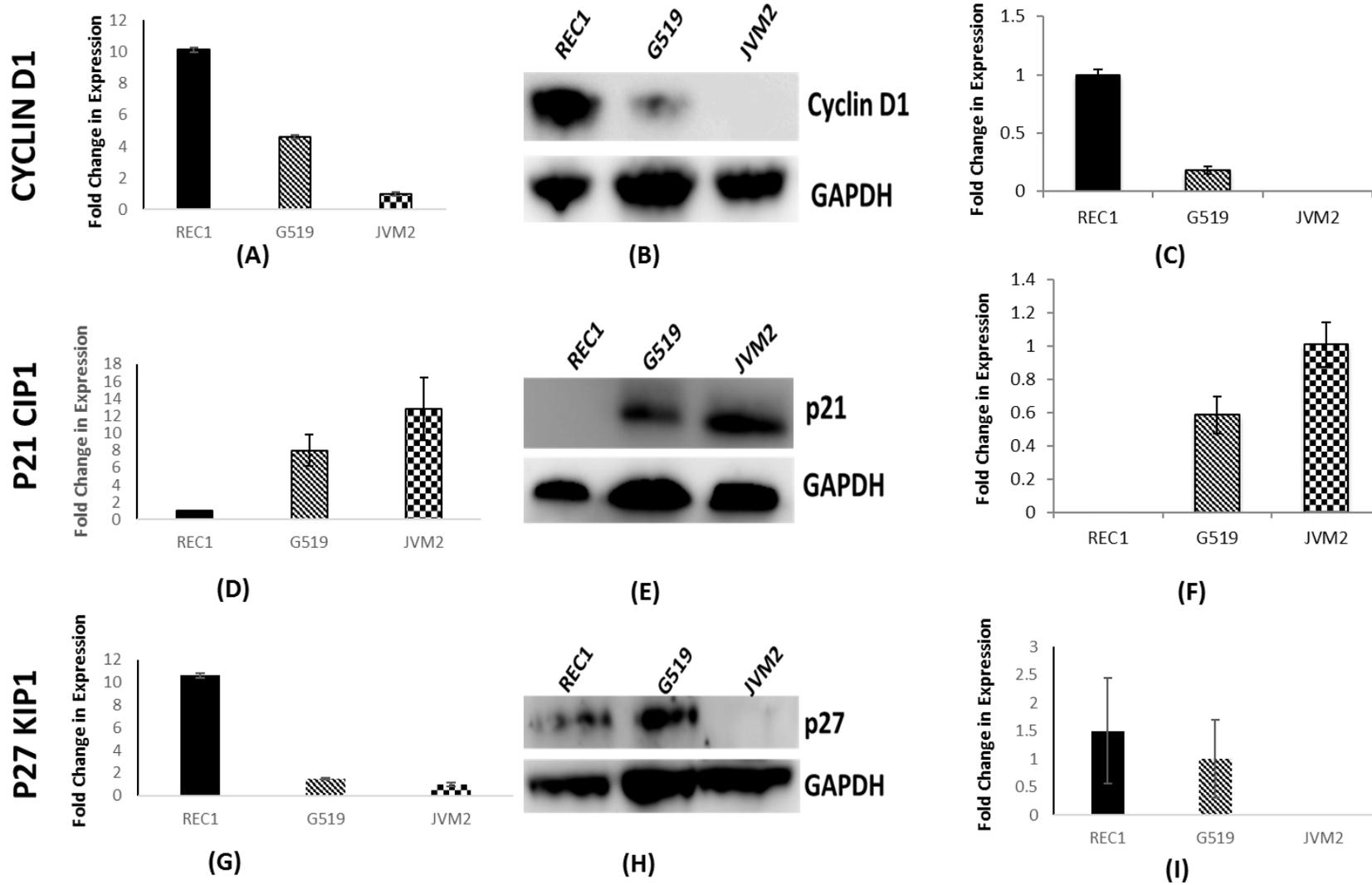


Figure 3

