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RESEARCH ARTICLE

PRO-PROLIFERATION PROWESS OF UPREGULATED JAKMIP1 IN HUMAN LUNG ADENOCARCINOMA

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ABSTRACT

Gaining insights into the possible functional aspects of relatively unknown genes to tumor genesis may help uncover new cancer biomarkers. Jakmip1, connected to autism spectrum disorders, is relatively new protein that we first demonstrated its ability to activate Wnt/beta catenin pathway and induce increased cervical carcinoma cell proliferation. In this report, we examined the impact of exogenous supplementation of Jakmip1 expression in lung adenocarcinoma. Cells over expressed for Jakmip1and their controls were assayed for their *in vitro* proliferation potential in CCK-8 and clonogenic assays, before they were implanted subcutaneously in nude mice to monitor tumor growth. Here, we present that up regulation of Jakmip1 increases cell proliferation and induce larger tumor development in xenografts. The acquisition of pro-proliferation prowess following Jakmip1 higher expression paralleled the protein expression of beta catenin and cyclin D1. Collectively, our data avidly suggest that upregulated Jakmip1 wields the potential to promote cancer growth and may be relevant to lung tumorigenesis.

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INTRODUCTION

Lung cancer still stands as the most common cancer globally, with adenocarcinomas holding some 40% share of cancers of the lungs (Lu *et al*., 2010). Generally, over the past few decades, search for biomarkers for early detection and effective treatment of cancers has been unrelenting. Paralleling this sticking point, a great interest in identifying biomarkers and signaling pathways mechanistically important to lung cancer progression has also been loudly apparent.

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The existence of challenges and limitations on cancer biomarkers in clinical use presently, means that relatively novel proteins ought to be examined for their relationship to the development of various cancers including that of the lungs. Janus Kinase and Microtubule Interacting Protein 1 (Jakmip1), alias Marlin 1, an interacting partner of Jak 1, Tyk 2 and microtubules, is comparatively new protein. Its expression is rich in brain and lymphoid tissues (Steindler *et al*., 2004) but moderate in lung, liver, muscle, retina (Costa et. al., 2007) and the testis (Vidal *et al.,* 2008). Jakmip1's structural organization, suggested involvement in cytoskeletal architecture (Steindler *et al.,* 2004) and predicted relevance to secretory pathway and transport machinery (Kohrmann *et al.,* 1999) in cells have precipitated interest. Recently decreased expression of Jakmip1 was observed in the cortex of FMR1 KO mice (Nishimura *et al*., 2007).

Also, Jakmip1 was reported to attenuate the cytotoxic ability of T lymphocytes (Libri *et al.,* 2008) and induced abnormal morphogenesis and migration in rat cortical pyramidal neurons (Vidal *et al*., 2012). Abnormal expression of Jakmip1 gene has been implicated in two genetic conditions linked to human autism (Nishimura *et al*, 2007; Bill and Geschwind, 2009). While its impact on various cell programmes and possible role in neurological disorders is gradually garnering attention, nothing is known of its significance to cell growth and proliferation. Jakmip1 interaction with Jaks has provoked the belief that it may have a role in Jak signaling pathways, an aspect of which-the Jak/Stat pathway-has been connected to the development of some cancers. Jakmip1functional relationship to cancer development has not been examined.

We previously reported that Jakmip1 is dysregulated in lung tumors and that when upregulated in a cervical cancer cell line, could induce Wnt pathway activity, enhance beta catenin build up and increase *in vitro* cell proliferation (Okai *et al.,* 2013). In our new study, we were interested in testing the proliferative power of increased Jakmip1 expression in a lung cancer cell line. We used both *in vitro* and *in vivo* models to examine Jakmip1's potential role in lung tumorigenesis. Now, we submit further evidence that, increased proliferation of cancer cells following Jakmip1 overexpression is not restricted to cervical cancer cells but even so for lung adenocarcinoma cells. Furthermore, in our xenograft model, Jakmip1 upregulation lead to the formation of larger tumours in nude mice, projecting increased expression of Jakmip1 as having potential oncogenic characteristics. Using immunoblotting techniques, mechanistic pathway studies of proteins, suggested that perhaps the means by which Jakmip1enhances cell proliferationis is by upregulating beta-catenin and cyclin D1 proteins.

MATERIALS AND METHODS

Plasmids, Cell Culture and Transfection

Plasmids were constructed as described elsewhere in our previous report (Okai *et al.,* 2013). Briefly, Jakmip1 promoters were PCR amplified from genomic DNA and then cloned in pcDNA3.1 expression vector and evaluated by gel electrophoresis and DNA sequencing. Lung adenocarcinoma cells, A549 were routinely cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Beyotime), and grown at 37^{o}C, 5% CO_2 in a humidified incubator. For transfection, cells were seeded in a 6-well plate at a density of $1x10^6$ cells per well and cultured to 90% confluence and 4µg of Jakmip1 plasmid or expression vector (pcDNA 3.1)alone was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

After 24 hours, the medium was replaced with normal medium. For the generation of stable cell lines, the transfected cells were selected with 250µg/ml of Geneticin 418. After selection, they were maintained in regular medium containing 200µg/ml G418. pcDNA3.1-Jakmip1-A549, pcDNA3.1-A549 andA549 cells were used for the various assays

Western Blot

Transfected cells in 6-well plates were harvested by trypsinization and cells collected by centrifugation before being treated with RIPA lysis buffer and protein samples prepared and denatured by boiling. Western blot was done as described elsewhere (Yang *et al*., 2012). Briefly, denatured protein samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane and blocked in 5% non-fat milk in 1% PBS for 1 hour. Membranes were incubated with the following antibodies - rabbit polyclonal JAKMIP1 antibody (Proteintech,1:500 dilution), mouse monoclonal beta-actin antibody (Proteintech,1:1000 dilution), mouse monoclonal cyclin D1 antibody (Proteintech, 1:500 dilution),HRP-conjugated goat anti-mouse (Thermoscientific, 1: 1000 dilution), HRP-conjugated goat anti-rabbit (Thermoscientific, 1:1000 dilution)-and imaged using Biorad ChemiDoc XRS after Enhanced Chemiluminescence (ECL) reaction.

Cell Proliferation and Cytotoxicity Assay

For cell proliferation assay, cells were seeded at a density of $5x10^2$ per well in a 96-well plate in triplicate. After the cells had attached usually after about 12 hours, 10ul of CCK-8 solution (Dojindo) in 100ul of culture medium was incubated with the cells for one hour and optical density (OD)-absorbance at 450 nm- was measured to obtain the OD at 0 hour. The proliferation rate was then checked by adding 10ul of CCK-8 to 100ul of medium and measuring the optical density at 24 hour, 48 and 72 hour periods, For each day, measurement were taken hourly for 4 hours.. For cytotoxicity assay, $5x10^3$ cell population was plated and treated with 25µM cisplatin for three days with measurements taken every 24 hours. The culture medium was replaced each day that OD was to be measured before adding CCK-8 solution. On addition of the CCK-8 solution OD was measured after three hours.

Colony Formation Assay

Cells were inoculated at $3x10^2$/well in a 6-well plate and grown for 5 days under optimal conditions of 37^{o}C, 5% CO_2 in a humidified incubator. Afterwards, the medium was discarded and the cells were washed twice with 1% PBS and then fixed in methanol for 15 minutes. They were washed again in PBS before 400µl of 5% crystal violet solution was added per well for 20 minutes and then washed off with PBS before colonies were counted. A group of cells numbering at least 50 was taken to represent one colony. Colony counts were done under the microscope (Olympus).

In Vivo Tumorigenic Assay

For our xenograft model, the method described by Seki *et al*. (2002) was adopted. In brief, cells were harvested by trypsinization, washed with 1% PBS and counted. Eight-week-old nude mice (Balb/c-nu) were injected subcutaneously with 1 x 10^6 cells suspended in 150 µl of HBSS. Each mouse received the same dosage at the lower right flanks. Mice were monitored for six weeks, after which they were sacrificed. The developed tumors were excised, and their dimensions (length

and width) and weight were recorded. Tumor volume was calculated as (Length x Width^2/2). The animal experimentation was conducted by following all relevant international, national and institutional guidelines and regulations.

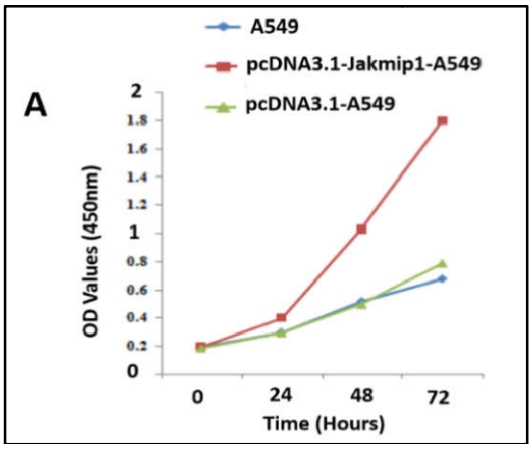
Statistical Analysis

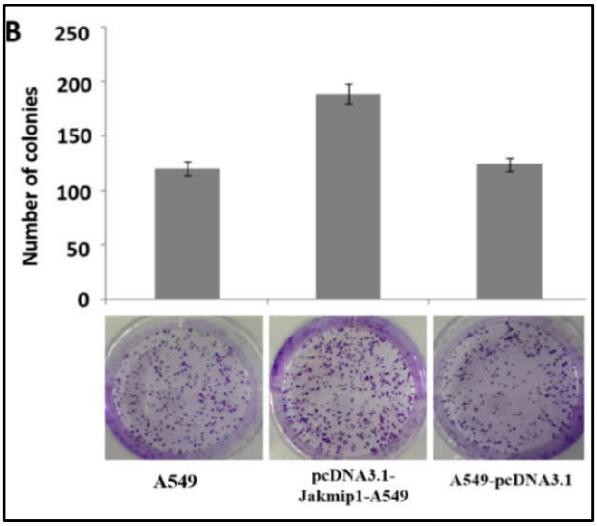
The results presented are the mean and standard deviation of three independent experiments. Students' unpaired t-test was performed to compare groups using SPSS 16 software. P-values less than 0.05 were considered statistically significant.

RESULTS

Jakmip1 Higher Expression Promotes Cell Proliferation

We increased Jakmip1 expression in the human lung adenocarcinoma cells, A549 to test the effect of such genomic augmentation on cell growth. CCK-8 cell proliferation assay demonstrated that Jakmip1 overexpression confers growth advantage. Cells harboring the recombinant Jakmip1 plasmid in culture grew at higher rate than the controls habouring empty plasmid or mock transfected. Mean OD recorded for pcDNA3.1-Jakmip1-A549 cells was significantly higher statistically ($p<0.05$) than those recorded for pcDNA3.1-A549 or A549 cells over the 72 hour period that growth of the test and control groups was monitored (Fig 1A).





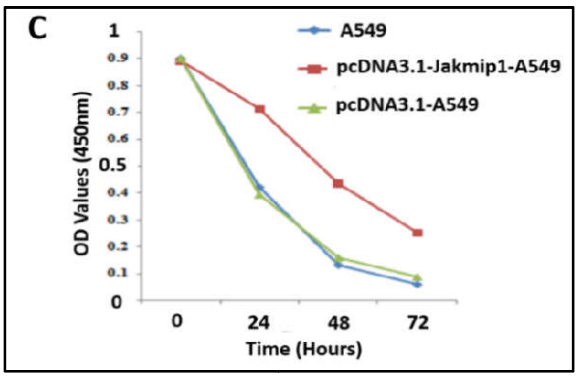


Figure 1. Overexpression of Jakmip1 increases cell viability and proliferation. A. CCK-8 cell proliferation assay. B. Clonogenic assay. C. CCK-8 cytotoxicity test for cell viability

No statistically significant difference in OD values was observed by comparing the control groups. To confirm this observation, we also performed colony formation assay and found that mean number of colonies formed by cells upregulated for Jakmip1 was significantly greater ($p<0.05$) relative to the cells without such upregulation (Fig1 B). When these two observations are put together, we believe that Jakmip1 overexpression gives some proliferative power to growing cancer cells

A549 Viability Is Enhanced By Jakmip1 Overexpression

In our attempt to examine if increased A549 cell proliferation post-Jakmip1 transfection was founded on cell viability, we carried out cytotoxicity test using cisplatin. CCK-8 cell viability assay revealed that cells overexpressing Jakmip1 were more viable than the untreated or empty plasmid bearing A549 cells (Fig 1C).

Mean OD values obtained for cells having higher expression of Jakmip1 was greater than those with controlled expression of the gene. Statistically significant difference ($p<0.05$) was observed between the test and controls. The better survival advantage borne by the Jakmip1 upregulated cells is consistent with the higher proliferative tendency seen in the colony formation and CCK-8 proliferative assays.

Elevated Beta-Catenin Expression parallels Jakmip1 Upregulation

Beta-catenin is major player in the canonical Wnt pathway which activates the expression of its downstream targets such cyclin D1 when there are Wnt ligands and its degradation is shirked. Its expression and the impact thereof on cell growth are important.

Thus, we used western blot to assess beta-catenin expression following Jakmip1 upregulation. Protein expression levels of beta-catenin were found to be higher in cells overexpressing Jakmip1 as against cells expressing empty plasmid or those untransfected (Fig 2). Apparently, increased expression of Jakmip1 protein induces higher expression of beta-catenin.

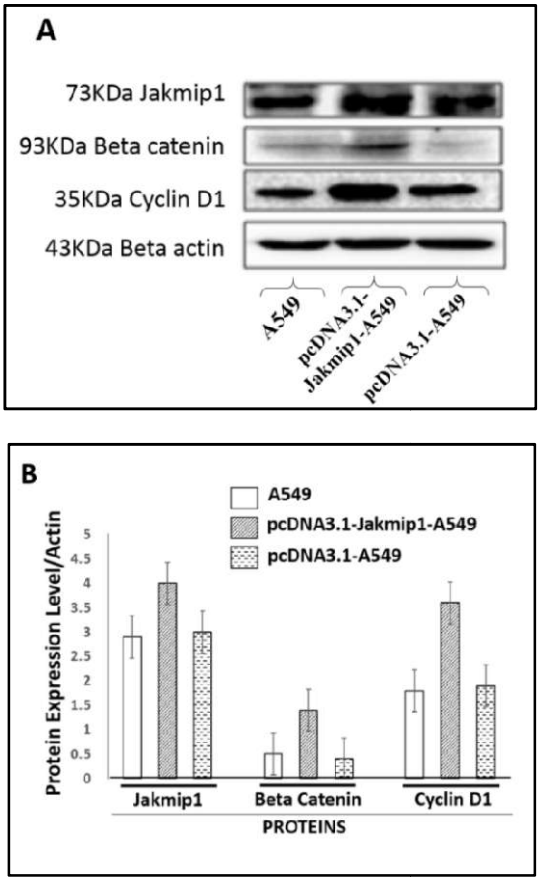


Figure 2. Increased expression of Jakmip1 elevates beta catenin and cyclin D1 protein expression. (A) Protein expression on immunoblot (B) Graphical illustration of protein level from western blot

Cyclin D1 Protein Expression Increases Upon Jakmip1 Overexpression

Cyclin D1 is a major regulator of G1/S-phase of the cell cycle and a loyal downstream target of beta-catenin, upon activation of the wnt/beta-catenin pathway. Beta-catenin activation positively regulates this protein. In our attempt to examine the effect of Jakmip1 upregulation of beta-catenin on cyclin D1, we resolved on SDS-PAGE and immunoblotted protein extracts of Jakmip1 upregulated cells and their controls. Consistent with the increased beta catenin expression upon Jakmip1 over-expression, we found that cyclin D1 expression was also amplified in cells upregulated for the recombinant Jakmip1 relative to the controls (Fig 2). Therefore, Jakmip1 upregulation of beta-catenin upstream correlates with the expression of downstream cyclin D1.

Jakmip1 Upregulation Induces Larger Tumor Development

In our quest to assess whether *in vitro* enhanced cell growth following Jakmip1 higher expression translates *in vivo*, nude mice inoculated with cells of the respective treatments were monitored for six weeks. In the xenograft model, mice habouring cells upregulated for Jakmip 1 developed larger tumors with greater tumor volumes than those with expression vector alone or were untransfected (Fig 3).

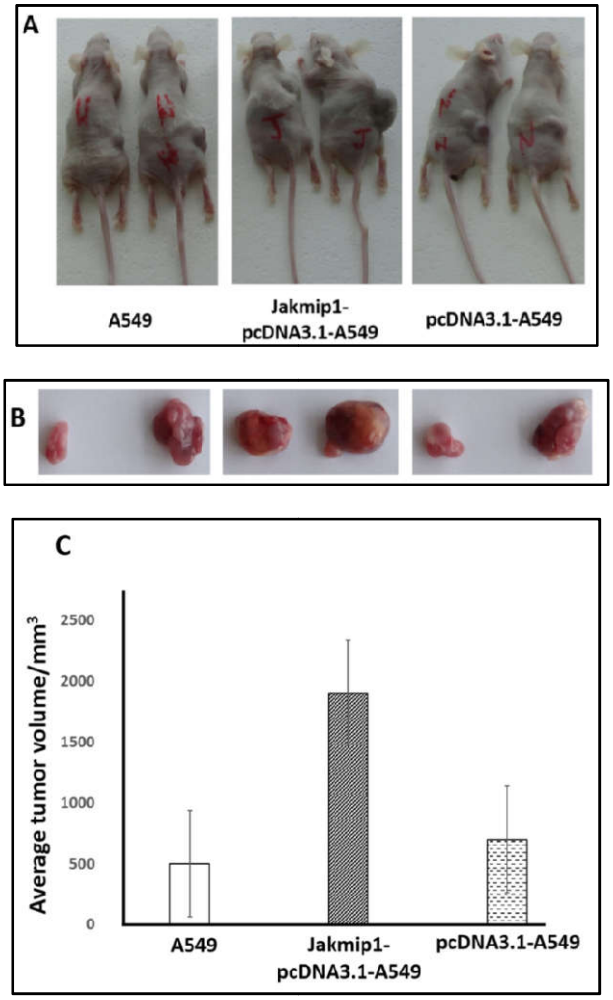


Figure 3. Overexpression of Jakmip1 induces larger tumors in xenografts. (A) Paired samples of nude mice with tumors (B) Excised tumors from nude mice (C) Graph of average tumor volume from excised tumors

The average tumor volume for the mice injected with cells overexpressing Jakmip1 was significantly greater statistically ($p<0.05$) than those of the mice with cells containing expression vector alone.

DISCUSSION

Despite the greater advances made in using various biomarkers for diagnostic, prognostic and therapeutic purposes, there are still limitations on the use of biomarkers in the clinical setting. This has driven immense interest in the continuous search of new biomarkers that have potential for clinical utility in cancer management. Several attempts have been made to elucidate the possible mechanistic roles of proteins aberrantly expressed in tumors. Jakmip 1 is comparatively new protein with scanty data on its relationship to diseases. A study by Nishimura *et al*. (2007) has implicated dysregulated Jakmip1 in autism spectrum diseases. Earlier, we submitted that *in vitro* upregulation of Jakmip1 apparently induced canonical Wnt pathway activity by increasing protein expression of beta catenin, and enhanced the proliferation of human cervical carcinoma cells. One of the key trademarks of cancers is increased cell proliferation (Bhatt, 2010), therefore, for a

relatively newly identified gene like Jakmip1, we were interested in knowing if its pro-proliferation characteristics seen in our earlier report was cancer type specific. Thus we developed both *in vitro* and xenograft models to study the impact of Jakmip1 overexpression in lung tumorigenesis. In our cell proliferation assays, it was observed that increased expression of Jakmip1 protein in the lung carcinoma cells resulted in higher rate of proliferation. Cells overexpressed for Jakmip-1 in CCK-8 and clonogenic assays grew faster than those lacking such genomic augmentation at a significant rate. This is consistent with our previous finding that Jakmip1 higher expression promotes faster growth of cervical carcinoma cells. This observation goes to suggest that the pro-proliferation potential of upregulated Jakmip 1 may not be restricted to particular cancer types. Having monitored the difference in cellular viability between Jakmip1 upregulated cells and the control by employing cisplatin-induced cytotoxicity, we observed that increased expression of Jakmip1 led to better cell viability. Cells' need for viability is an important requirement if they would be able to respond to any signals for growth and proliferation. Chronic cell proliferation is the most fundamental hallmark of cancers (Hanahan and Weinberg, 2011). Enhanced cell proliferation related to the overexpression of some genes has been implicated in many cancers. For example, *miR-17-92* cluster which is overexpressed in human lung cancers, promotes cell proliferation (Hayashita *et al*, 2005). The nexus between Jakmip1 overexpression and augmented cell propagation seen *in vitro* necessitated that we tested whether our observation is mimicked *in vivo*. We found from our xenograft experimental models that larger tumors were developed by mice subcutaneously implanted with cells upregulated for Jakmip1. There is the indication that *in vitro* induction of increased cell proliferation by Jakmip1 translates *in vivo*. Therefore, higher expression of Jakmip1 may be very relevant to the progression of lung cancers.

Earlier we reported that overexpression of Jakmip1 could induce canonical Wnt pathway activity with resultant accumulation of cytosolic beta catenin and enhance proliferation of cervical carcinoma cell lines *in vitro* (Okai *et al*., 2013). In this work, it was apparent that acquisition of more proliferative competency after Jakmip1 upregulation was linked to the expression of beta catenin and cyclin D1.Consistent with our previous report higher expression of Jakmip1 provoked increased expression of beta catenin and cyclin D1 (Fig. 2). Cyclin D1 protein functionally collaborates with CDK4 and CDK6 to drive cells through G1/S phase of the cell cycle (Baldin *et al*., 1993). However, its expression is greatly influenced by beta catenin, a key player of the canonical Wnt pathway. Elevated expression of beta catenin and cyclin D1, one of its downstream targets has been implicated in cancers (Morin, 1999; Diehl, 2002). Overexpression of cyclin D1 has been observed in cancers like the breast (Arnold and Papanikoulaou, 2005; Knudsen *et al*., 2006) and prostate (Knudsen *et al*., 2006). Increased beta catenin expression with concomitant increase in proliferation in acute lymphoblastic leukaemia cell lines after Wnt pathway activation with exogenous Wnt-3a has been noted (Khan *et al*., 2007). The expression of beta catenin and cyclin D1 in relation to higher proliferative activity of cancer cell lines is well documented (Mishina *et al*., 1999; Li *et al*., 2004). From our findings augmentation of Jakmip1 expression appears relevant to beta catenin and cyclin D1 activity in cancer cells. This ultimately goes to benefit these cells as it promotes their proliferation. Collectively, the data from this work suggest that abnormal expression of Jakmip1 may enhance tumor progression.

Conclusion

The evidences from our work strongly argue that overexpression of Jakmip1 promotes cell proliferation, and that this dedication to cancer cell propagation may be seen cancer type. Upregulated Jakmip1 appears to posses some oncogenic potential. Explorative studies on this relatively unknown protein may help to completely decipher its role in tumorigenesis and assess its prospects as a biomarker.

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Conflict of Interest

The authors declare that they have no conflicts of interest regarding this manuscript.

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