Final Report

Determination of microRNA profiles in the genesis of Opisthorchis viverrini-associated cholangiocarcinoma for chemopreventive and therapeutic applications

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รายงานวิจัย

เรื่อง

การตรวจสอบแบบแผนการแสดงออกของไมโครอาร์เอ็นเอในกระบวนการก่อมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดพยาธิใบไม้ตับเพื่อประยุกต์ใช้ในการป้องกันและรักษาโรค
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ทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ 2554
ชุดโครงการพยาธิใบไม้ตับและมะเร็งท่อน้ำดี

คำนำ

โครงการวิจัยนี้เป็นส่วนหนึ่งของชุดโครงการ “กลไกการก่อมะเร็งท่อน้ำดีในระดับโมเลกุลเพื่อการป้องกันด้วยเคมีอย่างมีประสิทธิภาพ” ซึ่งได้รับทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ **2554** (โครงการต่อเนื่อง **2554-2555**) ภายใต้การสนับสนุนครุภัณฑ์วิจัย วัสดุชีวภาพ (เนื้อเยื่อผู้ป่วยและเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี) และบุคคลากรของศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี มหาวิทยาลัยขอนแก่น

บทคัดย่อ

มะเร็งท่อน้ำดี เป็นโรคมะเร็งปฐมภูมิของเซลล์เยื่อบุผิวท่อน้ำดี สาเหตุเกิดจากการติดเชื้อพยาธิใบไม้ตับทำให้เกิดการอักเสบเรื้อรังซึ่งนำไปสู่กระบวนการก่อมะเร็ง การศึกษากลไกในระดับโมเลกุลของมะเร็งท่อน้ำดีจะทำให้ทราบโมเลกุลเป้าหมายที่จะนำมาประยุกต์ในการป้องกันและรักษาได้ วัตถุประสงค์ในการศึกษาครั้งนี้ เพื่อค้นหาแบบแผนการแสดงออกของไมโครอาร์เอ็นเอชนิด oncogenic microRNA โดยเกี่ยวข้องกับกระบวนการก่อมะเร็งท่อน้ำดีที่สัมพันธ์กับการอักเสบเรื้อรัง ผลการศึกษาพบว่า ไมโครอาร์เอ็นเอชนิด miR-21 มีการแสดงออกของ miR-21 เพิ่มขึ้นในเนื้อเยื่อตับของแฮมสเตอร์ที่ถูกเหนี่ยวนำให้เป็นมะเร็งท่อน้ำดี ในระยะ 2 เดือน (hyperplastic lesion) และ 6 เดือน (CCA) ยังพบว่า miR-21 เพิ่มขึ้นในเนื้อเยื่อมะเร็งท่อน้ำดีของผู้ป่วยและมีความสัมพันธ์กับการลดลงของโปรตีน PDCD4 การศึกษาด้วย siRNA ยับยั้งการทำงานของ miR-21 ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีพบว่า สามารถยับยั้งการเจริญเติบโตและการแพร่กระจายของเซลล์มะเร็งได้ และมีผลต่อระดับการแสดงออกที่เพิ่มขึ้นของ mRNA เป้าหมายจำนวนหลายชนิดได้แก่ ANKRD46, GLT8D3, TPRGL1, CDC25A และ YOD1 นอกจากนี้ยังพบว่า การลดลงของ miRNA-21 ทำให้ mRNA ในกลุ่มของ cell differentiation ได้แก่ STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1 และ SOX7 เพิ่มขึ้นอีกด้วย การศึกษาครั้งนี้สรุปได้ว่าเครือข่ายการทำงานของ miR-21 มีผลต่อการควบคุมกระบวนก่อมะเร็งท่อน้ำดีและการแพร่กระจายของเซลล์มะเร็ง หากสามารถยับยั้งการทำงานของโมเลกุลที่อยู่ในเครือข่าย miR-21 ได้ ก็น่าจะมีความเป็นไปได้ในการป้องกันและรักษาโรคมะเร็งท่อน้ำดีให้มีประสิทธิภาพดีขึ้นได้ในอนาคต

Abstract

Cholangiocarcinoma (CCA), a primary cancer of the bile duct epithelium, primarily associated with chronic infection with the liver fluke, *Opisthorchis viverrini* (Ov) that causes long-standing inflammation, the hallmark of carcinogenesis. Thus molecular mechanisms of CCA genesis and progression need to be studied in order to identify molecular targets for chemoprevention and treatment. Our study aimed to investigate the molecular pathways and the roles of an oncogenic microRNA, miR-21 which involve in chronic inflammation associated CCA progression. Our data showed that miR-21 expression was significantly upregulated in liver tissues of Ov plus NDMA treated hamsters at 2 months (hyperplastic lesion) and at 6 months when CCA had developed. MiR-21 was also upregulated in human CCA tissues and CCA cell. In patient's CCA tissues which miR-21 has been increased, we found that miR21 targeting genes such as the programmed cell death 4 (PDCD4) had weaker staining in CCA tissues compared to the normal biliary duct. Knocking down miR-21 caused the reduction of CCA cells growth and migration ability. Cells with suppressing miR-21 showed an increase in expressions of many genes including ANKRD46, GLT8D3, TPRGL1, CDC25A and YOD1. In addition, the gain-off of miRNA-21 expression related to the increased expression of STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1 and SOX7, which possibly controls cancer cell differentiation. Our data suggest that the molecular biology network of miR-21 might play role in the genesis Ov-associated CCA. Modulation of aberrantly expressed miR-21 may be a useful strategy to inhibit tumor cell phenotypes or improve response to chemotherapy including prevention of carcinogenesis.

Acknowledgment

This project was supported by Research Grant of Khon Kaen University 2011 under the auspice of Liver Fluke and Cholangiocarcinoma Research Center. We are also grateful to Faculty of Medicine, Khon Kaen University for all equipment support. Special acknowledgment is given to Prof. Bin Tean Teh for their great collaboration to get this work successfully done.

สารบัญ

รายงานวิจัย

เรื่อง

การตรวจสอบแบบแผนการแสดงออกของไมโครอาร์เอ็นเอในกระบวนการก่อมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดพยาธิใบไม้ตับเพื่อประยุกต์ใช้ในการป้องกันและรักษาโรค (Determination of microRNA profiles in the genesis of *Opisthorchis viverrini*-associated cholangiocarcinoma for chemopreventive and therapeutic applications)

ทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ 2554
ชุดโครงการ กลไกการก่อมะเร็งท่อน้ำดีในระดับโมเลกุลเพื่อการป้องกันด้วยเคมีอย่างมีประสิทธิภาพ

1. หน่วยงานที่รับผิดชอบงานวิจัย ภาควิชาชีวเคมี คณะแพทยศาสตร์
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โทรศัพท์/โทรสาร 043-348-386

2. คณะผู้วิจัย

1. หัวหน้าโครงการวิจัย ผศ.ดร. นิษณา นามวาท
2. ผู้ร่วมวิจัย รศ.ดร.พวงรัตน์ ยงวณิชย์
3. ผู้ร่วมวิจัย ผศ.ดร.วัชรินทร์ ลอยลม
4. ผู้ร่วมวิจัย นางสาวพรชีรา ชูสอน

3. เป็นส่วนหนึ่งของชุดโครงการ พยาธิใบไม้ตับและมะเร็งท่อน้ำดี

4. ประเภทของงานวิจัย งานวิจัยพื้นฐาน

5. สาขาวิชาการที่ทำการวิจัย วิทยาศาสตร์การแพทย์

6. คำสำคัญของเรื่องที่ทำการวิจัย มะเร็งท่อน้ำดี, cholangiocarcinoma, metastasis, miR-21

7. ความสำคัญและที่มาของปัญหาการวิจัย (Background and rational)

We have known for a long time that multistep processes of carcinogenesis had developed because of a result in the accumulation of several genomic alterations, unrestricted proliferation, invasion, and metastasis. In cancer, many molecular pathways are affected, involving canonical protein-coding genes as well as recently discovered noncoding genes. Noncoding RNAs include a class of small RNAs (~22 nucleotides in length), microRNAs (miRNAs) that modulate gene expression in either suppressing translation or degrading of target mRNA. Abnormal expressions of miRNA can contribute to tumor growth by modulating the expression of certain genes involved in carcinogenesis. Moreover, alterations of miRNA expression in cancer have encouraged the development of miRNA arrays to characterize the global miRNA changes in cancer, with the purpose of identifying specific miRNA signatures that can be used in the clinic for diagnostics, prognostics and for evaluation of treatment response. At present, there are many attempts using antagomiR to inhibit overexpression and agomiR to exhibit the low expression of endogenous miRNA involving in cancer. We also believe that intervention with either antagomiR or agomiR may increase chemosensitivity for treatment of cancer.

Chronic inflammation and infection are major causes of cancer. We are well known about the mechanisms of key mediators include nuclear factor kappa B, reactive oxygen and nitrogen species, inflammatory cytokines, and prostaglandins in inflammation-induced carcinogenesis. Interestingly, the current study demonstrated the linkage between inflammation, microRNAs and cancer. They demonstrated that inflammatory stimuli such as interleukin 1 and 6 (IL1, IL6), which always show high expression in inflammation-induced cancers, alter the expression of oncogenic-miRNAs including, miR-155, miR-146 and miR-21. Therefore, microRNAs have shown the role as mediators in inflammation-induced cancers.

Cholangiocarcinoma (CCA), is the cancer of bile duct caused by chronic inflammation of the liver fluke (*Opisthorchis viverrini*, Ov) infection. CCA is the major public health problem in northeastern, Thailand due to the high incidence of Ov in this area. Several reports of Ov associated CCA are generated but the genesis of CCA is still unclear. Therefore, the understanding of connections between inflammation, microRNAs and CCA may provide novel preventive, diagnostic and therapeutic strategies to reduce the health burden of this cancer. Modulation of aberrantly expressed microRNAs associated inflammation and CCA may be a useful strategy to inhibit tumor cell phenotypes or improve response to chemotherapy including prevention of CCA carcinogenesis.

Taken together of the above information, we hypothesize that in Ov infected bile ducts, generated the stimulation of inflammatory cytokines may trigger the dysregulation of microRNAs and activate the development of CCA. Therefore, we aim to investigate the expression profile(s) and the role(s) of miRNAs in the CCA carcinogenesis, in Ov associated CCA in both hamster and human. Moreover, we also propose whether microRNAs could be a potential target for CCA therapy by using of microRNA inhibitors in preventing the development and arresting the growth and spread of CCA by investigating mRNA targets upon modulation of aberrant microRNAs. We hope that the explored of microRNA expression profiles and intensive studies of their functions will be provided for the study of mechanism by which miRNAs regulate Ov-associated CCA carcinogenesis. Modulation of dyregulation of theses microRNAs will be benefit for inhibiting of carcinogenesis and treatment of this cancer.

8. วัตถุประสงค์ของโครงการวิจัย (Objectives)

<u>ปีที่ 1 (2554)</u>

8.1 To investigate the expression pattern of oncogenic miRNA (miR-21) in CCA carcinogenesis and to explore the molecular mechanism of miRNA in the control of cellular function

<u>ปีที่ 2 (2555)</u>

8.2 To analyze the expression profiling of miRNA and mRNA targets in human CCA tissues using miRNA microarray and bioinformatics analysis

9. ประโยชน์ที่คาดว่าจะได้รับ (Anticipated outcome)

9.1 The role of miRNA in CCA carcinogenesis

9.2 MiRNA as a targeted therapy for CCA

9.3 International publication and conference presentation

10. หน่วยงานที่จะนำเอาผลการวิจัยไปใช้ประโยชน์

1. คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

2. กระทรวงสาธารณสุข

11. งานวิจัยที่เกี่ยวข้อง (Literature review)

11.1 MiRNAs involved in inflammation and cancer

MicroRNA expression can be induced or expressed by a variety of mechanisms including direct transcriptional activation or repression from transcriptional enhancers, epigenetic modifications of the genome, genomic amplification or deletion as mentioned above, cellular stress and inflammatory stimuli (Schetter et al. 2010).

MicroRNAs also have an essential role in both the adaptive and innate immune system. Proper microRNA expression is required for correct differentiation of immune cells. In an innate immune response, specific microRNAs can be regulated by inflammatory stimuli and certain microRNAs can act as mediators of inflammatory stimuli (Lindsay 2008). Expression profiling experiments reveal that lipopolysaccharide (LPS) induced inflammation causes altered expression of several microRNAs including miR-146a, miR-132 and miR-155 in a human acute monocytic leukemia cell line (Moschos et al. 2007). Treatment with either of the pro-inflammatory cytokines, IL1β or TNFα, also stimulated the expression of miR-146a. The promoter region for miR-146a contains NFκB –binding sites, indicating that NFκB was probably responsible for driving the expression of miR-146a. Interestingly, miR-146a expression can inhibit interleukin-1 receptor-associated kinase and TNF receptor-associated factor 6, both of which are downstream factors involved in IL1 receptor signaling, demonstrating the first negative feedback loop involving microRNAs in an inflammatory response. Confirmatory studies found IL6 and IL8 to be negatively regulated by miR-146a through this feedback loop. Similar experiments were performed with miR-155 and demonstrated that this microRNA is induced by LPS or H. pylori infection and this is mediated by NFκB and activator protein-1. Increased miR-155 expression had a negative effect on IL8 signaling, indicating a role for miR-155in an inflammatory negative feedback loop (Schetter et al. 2010). Using a variety of inflammatory stimuli, many additional microRNAs have been shown to be linked to an inflammatory response, including miR-21, let-7, miR-9, miR-98, miR-214, miR-223, miR-224 and miR-513 (Gong et al. 2009; Hu et al. 2009).

Moreover, microRNA expression patterns are associated with chronic inflammatory diseases and other inflammatory condition. For example, psoriasis affected skin has increased expression of miR-203, miR-146a and miR-21 with reduced expression of miR-125b (Sonkoly et al. 2007). Lung inflammation due to allergic reactions also increased miR-21 expression (Lu et al. 2009). Rheumatoid arthritis, a chronic inflammatory autoimmune condition affecting joints and tissues found elevated levels of miR-146a (Nakasa et al. 2008). MicroRNAs are also altered in inflammatory conditions that are associated with increased risk of cancer. Such as, primary biliary cirrhosis, a chronic inflammatory autoimmune condition of the bile duct, carries an increased risk of liver cancer. In this condition, miR-122a and miR-26a were reduced and miR-328 and miR-299-5p were increased (Padgett et al. 2009). These changes in microRNA expression levels may contribute to both the active inflammation and the increased risk of cancer associated with these diseases. Role of microRNAs in inflammation is summarized in **Figure 11.1.**

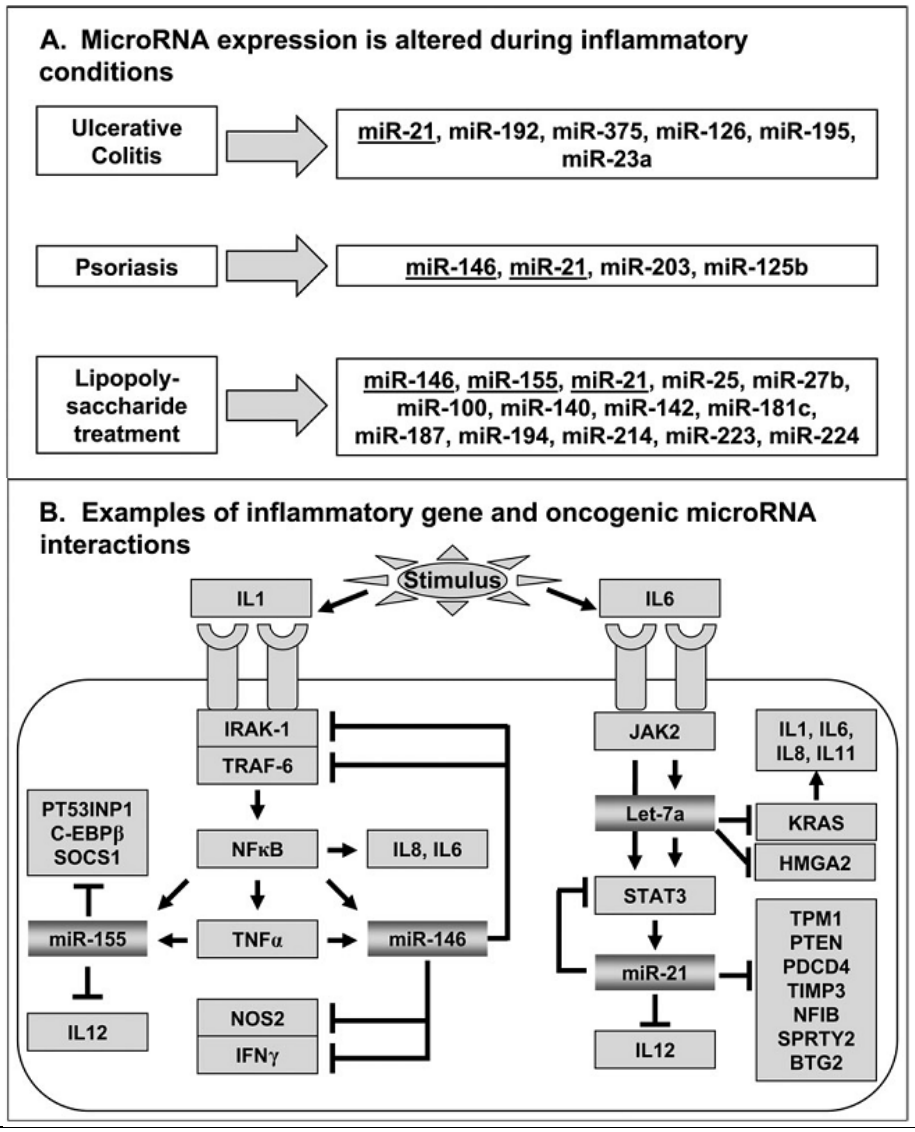


Figure 11.1 MicroRNAs are inflammatory mediators. (A) MicroRNA expression levels are altered in a variety of inflammatory conditions (Schetter et al. 2010).
Inflammatory stimuli alter the expression of microRNAs while specific microRNAs have oncogenic or tumor-suppressive activity. Therefore, microRNAs may be mediators of inflammation-induced carcinogenesis.

Inflammatory stimuli can increase the expression of miR-21. Such as IL6, a pro-inflammatory cytokine, can induce the expression of miR-21 in a STAT3 dependent manner (Loffler et al. 2007). The EGFR pathway has also been shown to increase miR-21 expression (Seike et al. 2009). Increased levels of miR-21 are also found in several chronic inflammatory diseases, including asthma (Lu et al. 2009), ulcerative colitis (Wu et al. 2008) and Crohn's disease (Wu et al. 2010). The elevated levels of miR-21 in these tissues may be inpart responsible for inflammation-associated cancers. Increased levels of miR-21 are found in nearly every malignancy examined and this increase is summarized to be oncogenic miRNA (Selcuklu et al. 2009).

Recently, miR-21 was found to directly target and repress IL12-p35 expression in mouse models (Lu et al. 2009). The miR-21-binding site in the 3' UTR of IL12-p35 is conserved in humans. Moreover, the study of (Schetter et al. 2009) have indicated that miR-21 expression negatively correlates with IL12-p35 expression, consistent with the idea that

IL12-p35 is a target for miR-21, and positively correlates with IL6 expression in human colon cancer tissues, consistent with IL6 driving the expression of miR-21. This suggests that the *in vitro* data demonstrating the connections between IL6, IL12-p35 and miR-21 are probably relevant in the context of human colon cancer and that miR-21 may contribute to inflammation-induced carcinogenesis. MiR-21 targets and functions will be discussed in 11.2.

11.2 MiRNA-21 expression and function in cancer

MiR-21 was the only miRNA up-regulated in all types of the analyzed tumor samples or cancer cell lines as shown in **Table 11.1** (Krichevsky and Gabriely 2009). In addition, another study showed that overexpression of miR-21 in primary breast cancer samples is associated with advanced clinical stage, lymph node metastasis and poor prognosis (Yan et al. 2008). Moreover, a novel RT-qPCR-DS (RT-qPCR applied directly in serum) detected circulating miR-21 has diagnostic and prognostic potential in breast cancer (Asaga et al.).

Table 11.1 Overexpression of miR-21 in various types of cancers (Krichevsky and Gabriely 2009)

Cancer	miR-21 expression in human tissues/cells	miR-21 involvement in biological process	miR-21 targets
Glioma	Up-regulation in GBM tumours, glioma cell lines	Invasion and cell growth	PDCD4, RECK, TIMP3?, NFIB, APAF1?, STAT3?
Breast cancer	Up-regulation	Cell growth, apoptosis, angiogenesis and invasion	PDCD4, TPM1, maspin
Ovarian cancer	Up-regulation		
Colorectal cancer	Up-regulation	Cellular outgrowth, migration, invasion and metastasis	PDCD4, NFIB, SPRY2
Stomach/gastric cancer	Up-regulation		RECK
Hepatocellular carcinoma	Up-regulation	Cell migration and invasion and proliferation	PTEN?
Cholangiocarcinoma	Up-regulation		PTEN?

Although it is known that miR-21 is overexpressed in cancer cells/tissues and has oncogenic activity in terms of neoplastic transformation, little is known regarding the genes and pathways downstream that are regulated by miR-21, particularly those that, if misregulated, can trigger neoplastic cellular growth.

Much of the early efforts to identify downstream targets of miRNAs have been based on computational target prediction algorithms, and the number of predicted miRNA target genes vastly outnumbers the number of miRNA target genes that have been actually validated in wet-lab experiments. This is evident from the fact that there are over 600 known miRNAs potentially (i.e. based on computational screens) targeting up to 30% (more than 5300 genes) of protein-coding genes in the human genome, yet there are only 461

experimentally validated miRNA–target gene demonstrations in TarBase (Selcuklu et al. 2009).

A number of tumour-suppressor genes have been found to be targeted by miR-21, supporting its proposed oncogenic role in cancer **(Figure 11.2)**. Validation studies of miR-21 targets prefer to use techniques including two-dimensional proteomics, luciferase reporter analysis and Western blot assays to screen for translational suppression of target genes. A number of tumor suppressor genes including programmed cell death protein 4 (PDCD4) (Chen et al. 2008; Yao et al. 2009), tropomyosin 1 (TPM-1) (Zhu et al. 2007), Sprouty2 (SPRY2) (Sayed et al. 2008), mammary serine protease inhibitor (Maspin), apoptotic protease activating factor-1 (APAF1) (Krichevsky and Gabriely 2009), phosphatase and tensin (PTEN) homologue (Zhang et al. 2010), a tissue inhibitor of MMPs that inhibits angiogenesis and tumor cell infiltration and induces apoptosis (TIMP3) (Gabriely et al. 2008) and reversion-inducing cysteine-rich protein with kazal (RECK) motifs (Zhang et al. 2008) are validated as targets of miR-21. In addition, an indirect regulation of Bcl-2 by miR-21 has also been shown in breast cancer.

Interestingly, miR-21 seems to be involved in a number of positive and negative feedback loops, and therefore is a part of the complex regulatory network operating in both normal and diseased cells **(Figure 11.2)**. These complex regulations may explain why miR-21 is probably one of the most dynamic miRNAs responsive to various stimuli.

(Fujita et al. 2008) demonstrated NFIB, a transcriptional repressor suppresses basal expression of the miR-21 gene and AP-1 mediates transcriptional activation of the miR-21 promoter. In addition, miR-21 knockdown in glioma cells leads to down-regulation of c-Jun mRNA (Gabriely et al. 2008), suggesting that miR-21 indirectly activates expression of c-Jun and thus may also induce AP-1-dependent transcription. Since AP-1 itself acts as a miR-21 inducer in cancer cells, it can in fact initiate the self-perpetuating circle of AP-1-dependent transcription of cancer genes. STAT3-dependent miR-21 transcription has been demonstrated in several cell types (Loffler et al. 2007), and it may be one of the factors inducing miR-21 expression in some cancers (Schetter et al. 2010). MiR-21, in turn, may down-regulate STAT3, since it has two conserved miR-21 binding sites, and STAT3 mRNA levels were regulated in glioma cells by both miR-21 inhibition and overexpression. Such a regulatory loop between miR-21 and IL-6/STAT3 may provide a feedback mechanism for stabilizing miR-21 expression and balancing STAT3 signaling.

Further work is also required to explore the potential relationship between miR-21 and TGF-β signaling because miR-21 maturation is induced by TGF-β and BMP4 ligands (Hata and Davis 2009). Microarray analysis of (Gabriely et al. 2008) demonstrated that miR-21 may regulate, either directly or indirectly, TGF-β, BMP4 and EGF factors, as well as receptors TGF-β R1 and TGF-β R2 (that are predicted as direct miR-21 targets).

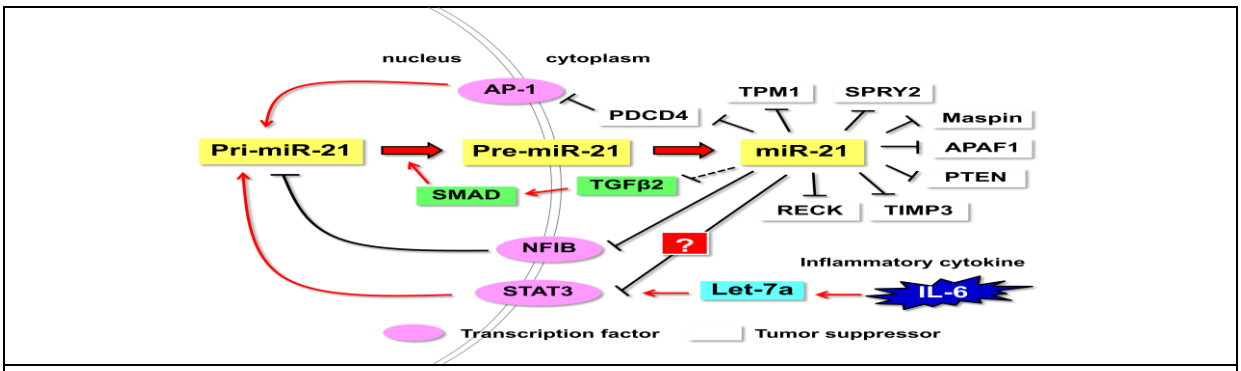


Figure 11.2 Model of miR-21 network and feedback regulation (Krichevsky and Gabriely 2009; Schetter et al. 2010; Selcuklu et al. 2009)

11.3 MiRNAs in CCA

Recently (Meng et al. 2006) showed that miR-141 was highly overexpressed in malignant cholangiocytes . Using a bioinformatics approach, a predicted target of miR-141 was the CLOCK gene, which regulates circadian rhythms and can act as a tumor suppressor. Inhibiting miR-141 effectively increased CLOCK protein expression in cholangiocarcinoma cells. Another microRNA species that was found to be overexpressed in malignant cholangiocytes was miR-200b. The target gene for this was predicted to be the protein tyrosine phosphatase non-receptor type 12 (PTPN12), the dysregulation of which may contribute to tumor cell survival and oncogenesis. Similarly, the expression of miR-21 was overexpressed in cholangiocarcinoma, which effectively blocks the expression of the tumor suppressor gene PTEN.

Conversely, other microRNA species have been identified as being downregulated in CCA compared to non-malignant cholangiocytes. MiR-29b expression was suppressed in the CCA cell line KMCH (Mott et al. 2007). Enforced miR-29b overexpression in CCA cells effectively reduced the expression of Mcl-1, an anti-apoptotic protein of the Bcl-2 family and sensitized CCA cells to tumor necrosis factor-related apoptosis-inducing ligand cytotoxicity, suggesting that the suppression of miR-29b expression found in CCA allows the overexpression of Mcl-1 and can ultimately lead to the resistance of CCA to cell death. Moreover, transfection of non-malignant cells (that express high levels of mir-29) with a locked-nucleic acid antagonist of mir-29b could increase Mcl-1 levels and reduced TRAIL-mediated apoptosis (Mott et al. 2007).

Another microRNA that is downregulated in CCA is miR-370 (Meng et al. 2008). Interestingly, the expression of this particular microRNA has been shown to be under tight epigenetic regulation by hypermethylation. One of the targets for miR-370 is the oncogene mitogen-activated protein kinase kinase kinase 8 (MAP3K8), thus MAP3K8 is upregulated in CCA cell lines as well as in tumor cell xenografts *in vivo* (Meng et al. 2008).

In addition to changes in promoter methylation, aberrant IL-6 expression in CCA also has implications on microRNA expression and function. Enforced IL-6 overexpression in human CCA cell lines significantly increased the expression of several members of the let-7 family of microRNAs (Meng et al. 2007). Expression of let-7a contributes to the survival effects that are attributed to IL-6 over expression. A putative target of let-7a is the gene neurofibromatosis 2 (NF2), which is a negative regulator of Stat-3. Thus, overexpression of IL-6 in CCA and subsequent upregulation of let-7a decreased the expression of NF2, thereby removing the negative regulation of Stat-3 (Meng et al. 2007). Constitutive activation of Stat-3 has been implicated in a number of cancers and is thought to be responsible for the IL-6-mediated survival signaling. The reports of microRNAs in CCA have been summarized in Table 11.2.

Table 11.2 MicroRNAs in CCA studies

Sample	miR profile	Xenograft	Candidate miRs	Level in CCA	Validated targets	Function	Combined drugs	Results	Ref
CCA cell lines			miR 200b miR 21 miR 141		PTPN12 PTEN CLOCK	Tumor growth	Gemza (knock - miR)	- "Cholangiocyte - miR cluster" - Gemcitabine could elevate miR-21 expression in vivo (miR-21 might be involved in drug resistance) - Anti-miR 21 & 200b increased gemcitabine-induced cytotoxicity.	(Meng et al. 2006)
CCA cell lines & tissues			miR 21		PDCD4 TIMPT3	Apoptosis Metastasis		- miR 21 accurately distinguishes between CCA & normal bile duct.	(Selaru et al. 2009)
			miR 370 miR 198 miR 560						
CCA cell lines & tissues			miR 21					- miR expression were related to clinicopathological classification	(Selaru et al. 2009)
			miR 320 miR 204		Mcl-1 Bcl-2	Anti-apopotosis			
CCA cell lines (KMCH)	Specific		miR 29b		Mcl-1	Anti-apoptosis	TRAIL (over miR)	- Enforced miR-29b expression reduced Mcl-1 & sensitized cancer cells to TRAIL cytotoxicity.	(Mott et al. 2007)
Mz-IL-6			miR 21 Let-7a		NF-2	Inhibitor of STAT3		- IL-6 can increase let-7a expression (low NF-2 but high STAT3 protein levels)	(Meng et al. 2007)
Mz-IL-6			miR 370		MAP3K8	Tumor growth	5-aza-CdR	- IL-6 can increase methylation activity and reduce miR-370 expression.	(Meng et al. 2008)

12. ระเบียบวิธีวิจัย (Research methodology)

12.1 Hamster tissues

Induction of CCA in male Syrian golden hamsters was performed in our laboratory by Ov metacercariae infection combined with *N*-nitrosodimethylamine (NDMA) treatment. The animal experiments were conducted according to the guidelines of the National Committee of Animal Ethics. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand. In brief, out-bred Syrian golden hamsters (*Mesocricetus auratus*), ranging from 6 to 8 weeks of age and

weighing approximately 100 g, were arbitrarily divided into 2 groups: Group 1 remained untreated; Group 2 was infected with 50 Ov metacercariae and treated with NDMA (Sigma, St. Louis, MO) (12.5 ppm given in drinking water) for 8 weeks. Each hamster received approximately 0.166-0.04 mg/day of NDMA. After treatment, five animals per group were sacrificed at weeks 1, 4, 12 and 24. Liver tissue from each animal was fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin for histological and immunohistochemical examinations.

12.2 Patients and samples

Thirty paraffin-embedded and 30 frozen intrahepatic CCA specimens from primary tumors of patients, collected between 2002 to 2004, are obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision. Human Research Ethics Committee, Khon Kaen University (#HE43201 and #HE471214), approved the research protocol.

12.3 CCA cell lines

Two types of cell lines are studied: (1) human intrahepatic CCA cell lines M156, KKU-M213, KKU-M214, KKU-M055, KKU-M139 and KKU-OCA17 established at Khon Kaen University Liver Fluke and Cholangiocarcinoma Research Center.

12.4 Total RNA extraction by TRIZOL® Reagent (Invitrogen, CA, USA)

Total RNA was isolated from liver tissues (approximately 100 mg) following the manufacturer's protocol (Invitrogen). Briefly, adding 1 ml of TRIZOL® Reagent into tissues and homogenize. The lysate was then incubated for 5 min at 15-30 ^{o}C to permit the complete dissociation of nucleoprotein complex. Then, 0.2 ml of chloroform is added to the mixture and a tube was shaken vigorously by hand for 15 sec, followed by incubation at 15-30 ^{o}C for 3 min. The mixture was centrifuged at 13,000 rpm for 15 min at 2 to 8 ^{o}C. After centrifugation, aqueous phase was transferred to a new 1.5 ml microtube. After that, RNA was precipitated by adding 0.5 ml isopropanol, and then incubated for 10 min at 15-30 ^{o}C and then the mixture was centrifuged at 13,000 rpm for 10 min at 2 to 8 ^{o}C. After centrifugation, supernatant is removed and washed RNA pellet once by adding 1 ml of 70% ethanol and then centrifuged at 13,000 rpm for 5 min at 2 to 8 ^{o}C. Briefly, the RNA pellet was air-dried, re-dissolved by adding 30-300 µl of RNase free water, and then stored at – 70 ^{o}C until used.
The 85% (v/v) ethanol is used instead of 70% (v/v) ethanol to wash the RNA pellet for miRNA study. Additionally, 10–15 µg of glycogen (Ambion) is added as a carrier prior to precipitation.

12.5 Total RNA extraction by mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA)

The mirVana™ miRNA Isolation Kit were used for purification of RNA studies of human CCA tissue or cell lines (10^6 cells). The kit employs an organic extraction followed by immobilization of RNA on glass-fiber filters to purify either total RNA, or RNA enriched for small species.

12.6 Transient knocking down of miR-21

High to moderate endogenous miRNA expression, CCA cells (3x 10^5 cells) were seeded into a 75 cm^3 flask for 48 hr before transfection. The anti-miR^{TM} miRNA inhibitor specific for miR-21 and validated non-targeting siRNAs (scramble controls) (Ambion) were transfected using RNAiMAX Transfection Agent (Invitrogen), according to the manufacturer's instruction.

12.7 Quantitative Real-Time-PCR (qRT-PCR)

The candidate miRNAs taken from the miRNA array data are validated by SYBR Green I qRT-PCR analysis. For this, the cDNA from each candidate miRNA was

synthesized using the reverse transcription with oligodA_s as a primer. PCR was performed by adding predesigned miRNA specific primer and a universal primer supplied by the manufacturer (Invitrogen). RNU49 snRNA was used as an internal control. Relative expression is calculated using the $\Delta\Delta C_T$ method**.**

12.8 Western blot analysis

After transient knocking down of miRNA, cells are harvested, protein lysates are prepared using protein lysis buffer that containing 30mM Tris, 7M Urea, 2M Thiourea, 4% (w/v) CHAPS and Protease inhibitor cocktail (Roche). Protein extracts are quantified using the Pierce BCA™ Protein Assay Kit (Pierce Biotechnology). Then, protein extracts are electrophoresed by 10% (w/v) SDS-PAGE, transferred to a PVDF membrane (Whatman), then incubated overnight with 5% (w/v) non-fat dry milk. The blots are probed with specific primary antibodies and anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA) as an internal control. After that, the membranes are incubated with secondary antibodies at room temperature for 1 hr, and proteins are detected by the Enhanced Chemiluminescence Plus solution (GE Healthcare). The apparent density of the bands on the membranes was captured by ImageQuant Imager and analysed using ImageQuant analysis software (GE Healthcare).

12.9 Immunohistochemical staining

Target proteins were detected on the paraffin-embedded sections using standard immunohistochemistry protocols. In brief, 4-μm-thick sections were incubated with appropreciate dilution of anti-human antibody for 1 h in a humidified chamber at room temperature. Sections were incubated with peroxidase-conjugated Envision secondary antibodies and peroxidase activity was visualized with DAB solution. Hematoxylin was employed for counterstaining. Staining frequency of proteins was semi-quantitatively scored based on percentage of positive cells as follows: 0% = negative; 1%-25% = +1; 26%-50% = +2; and >50% = +3. The intensity of protein staining was scored as weak = 1, moderate = 2, and strong = 3. For determination of biliary epithelial hyperplasia and dysplasia, hyperplastic ducts were defined as having increased cell numbers, enlargement of duct size and pseudopapillary protrusion, while dysplastic ducts were defined as having cells with multilayered nuclei, increased nuclear-cytoplasmic ratio and micropapillary projections.

12.10 Microarray printing, labeling, and hybridization

Microarray technique is used to analyze the miRNA expression profile from FFPE liver tissue, using Agilent™ microRNA Arrays (Agilent). Briefly, Total RNA (2–4 μg) is 3′-end-labeled using T4 RNA ligase and a Cy3-labeled RNA linker by the following procedure. The RNA in 4.5 μL of water is combined with 0.8 μL of T4 RNA ligase buffer (10X), 1.1 μL of polyethyleneglycol (50% [w/v]), 0.8 μL of RNA-linker (250 μM), and 0.8 μL of T4 RNA ligase. The reaction is incubated for 2 h at 30°C, and then terminated by incubation for 3 min at 80°C. Labeled RNA (8 μL) is combined with 6 μL of 20× SSC, 1.5 μL of herring sperm DNA (10 mg/mL; Roche), 11.4 μL of formamide, 0.6 μL of 5% SDS, and 2.5 μL of DEPC-treated water. Samples are denatured for 1–2 min at 80°C and hybridized to the microarray for 16–20 h at 65°C under a lifterslip. Post-hybridization washes are in 4X SSC at 60°C to remove the coverslip, followed by three times in 2X SSC, 0.025% SDS for 5 min each, three times in 0.8× SSC for 2 min each, and two times in 0.4X SSC for 3 min each.

12.11 mRNA array and data analysis

KKU-M214 and KKU-100 cells were transfected with antimiR-21 at a final concentration of 30 nM. Total RNAs were isolated from cells 24 h post-transfection using QIAgen mini RNAesy kit (QIAgen). The mRNA expression profile was performed using human genome Affymetrix oligo array core facility service (Singapore). Each sample was analyzed once, and the core facility data preprocess, normalization and filtering were done. Ratios were defined as marginal signal intensity when there was a substantial amount of

variation in the signal intensity within the pixels from 800 to 1,500. Candidates of miR-21 targets were determined by several algorithms including miBridge (licensing software), TargetScan, PicTar and miRTarbase.

12.12 Cell growth assay using MTT

The number of cells in each well after treatment (48 hours) with anti-miR-21 was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT labeling reagent (final concentration, 0.5 mg/ml) was added to each of CCA cells, (2×10^3 cells/well) in 96-well culture plates (final volume, 100 µl culture medium/well) and incubated for 4 hours at 37°C in a humidified atmosphere of 10% CO_2. Subsequently, cells were incubated overnight with 100 µl of solubilization solution per well, and the samples were quantified at 570 nm using a microtiter plate reader.

12.13 Statistical analysis

Target gene expression in human CCA tissues and adjacent non-tumorous tissues were compared using Wilcoxon matched pair test. Clinicopathological characteristics were compared with target gene expression (high and low) using Chi^2 or Fisher's exact probability test. Kaplan-Meier method was used to calculate survival curves, and Log-rank test was performed to compare differences in the survival rates of patients who were subjected to surgery. A multivariate analysis was performed by the Cox proportional hazard regression model. All analyses were performed using SPSS software (version 15.0). $P \leq 0.05$ was considered significant.

13. ผลการวิจัย (Results)

13.1 The miR-21 is upregulated in liver tissue as CCA develops

The miR-21 expression was found to be elevated after Ov plus NDMA administration in hamsters on week 1 and thereafter. The miR-21 levels were remarkably upregulated in hamsters from 3 weeks until 24 weeks when the CCA developed (Fig. 2). The miR-21 level was elevated in hyperplasia (ON2M) -> dysplasia (ON3M, ON4M) and the level was highest at tumorous stage (ON6M). The miR-21 level was increased in Ov or NDMA treated group but the level was lower than that in Ov+NDMA (Fig. 13.1).

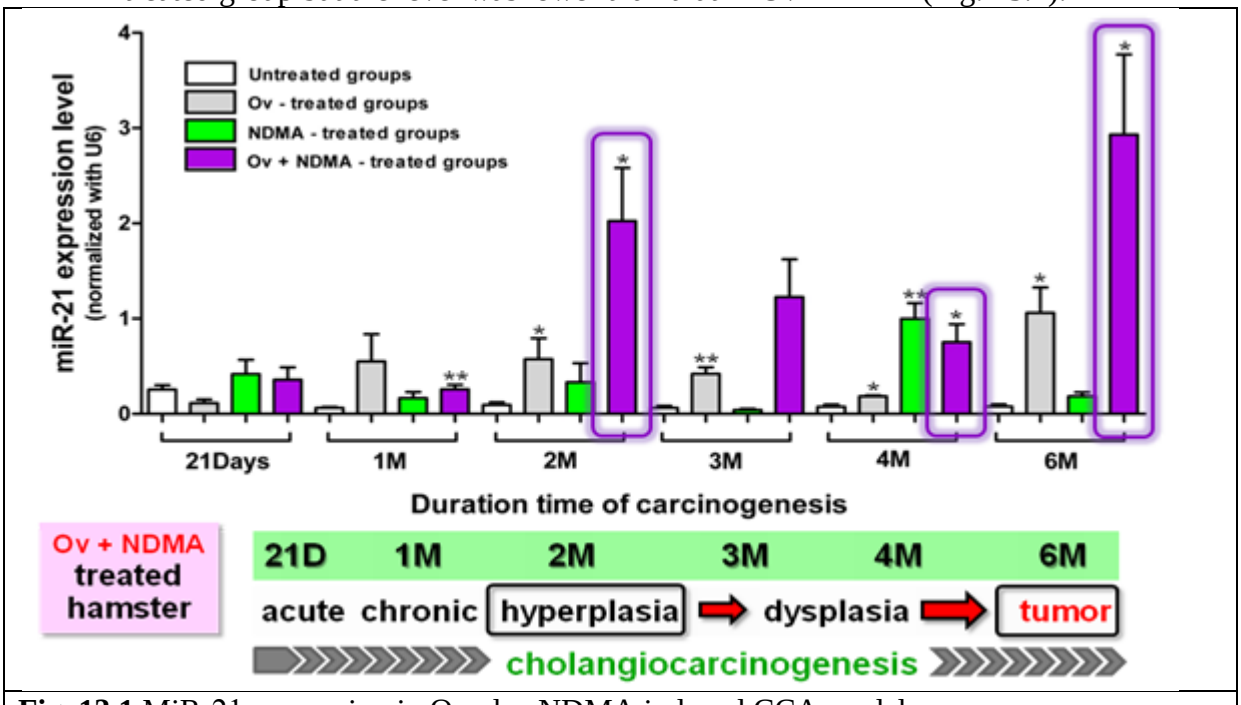


Fig. 13.1 MiR-21 expression in Ov plus NDMA induced CCA model

13.2 The miR-21 is upregulated in human CCA tissues

Analysis by qRT-PCR of 30 matched cases of human intrahepatic CCA tissues demonstrated that miR-21 expression was significantly up-regulated in tumor tissues as opposed to their adjacent non-tumorous tissues ($P < 0.001$, Wilcoxon matched pair test) (Figure 13.2).

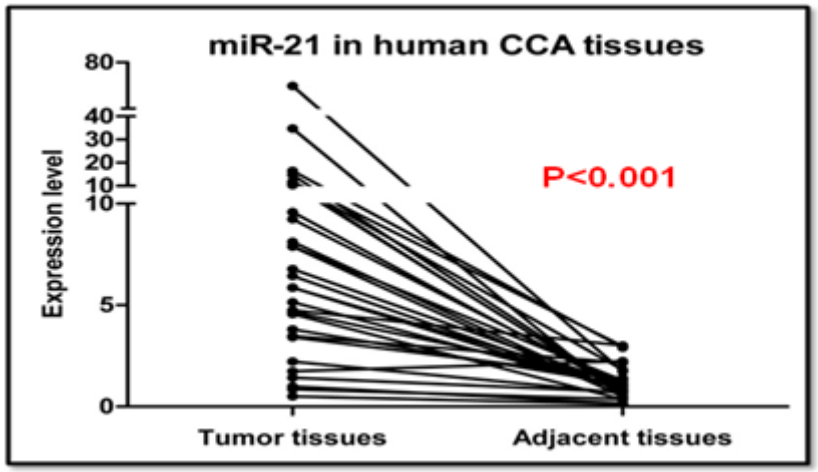
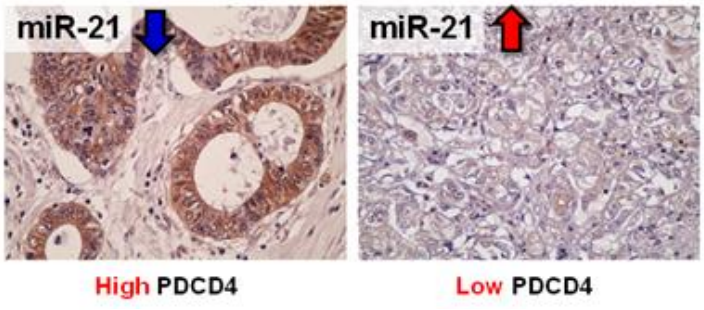


Fig. 13.2 MiR-21 expression in tumor and non-tumor tissues of CCA patients.

13.3 The miR-21 expression pattern was inversely correlated with PDCD4 proteins in human CCA

Among 24 cases, the result demonstrated that high level of miR-21 was appeared in cases with weak staining of PDCD4 (P=0.032). On the other hand, low level of miR-21 was appeared in cases with strong staining of PDCD4 (P=0.032) (Fig 13.3).



MIR21* PDCD4 Crosstab

		PDCD4		
		low PDCD4	high PDCD4	Total
MIR21	low miR-21	3	7	10
	high miR-21	11	3	14
Total		14	10	24

Fig 13.3 PDCD4 staining in tumor tissues of CCA patients (24 cases). The Fisher Exact test showed the negative correlation between miR-21 and PDCD4 (P=0.032).

13.4 Knocking down miR-21 suppressed CCA cell growth

CCA cell lines, KKU-M214 and KKU-100, were transfected with anti-miR-21. After 48 hr incubation, the decreased level of miR-21 was observed (Fig 13.4). Cell viability was decreased in both cell lines (Fig. 13.5).

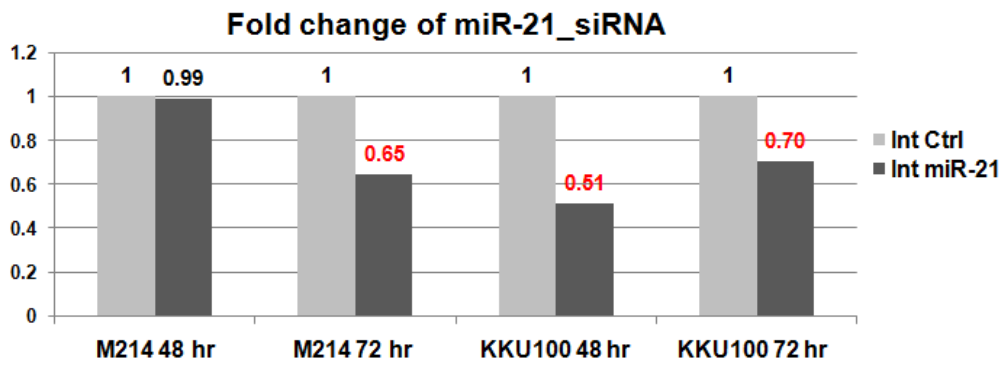


Fig 13.4 The decreased level of miR-21 in transient knock down CCA cell lines, KKU-M214 and KKU-100

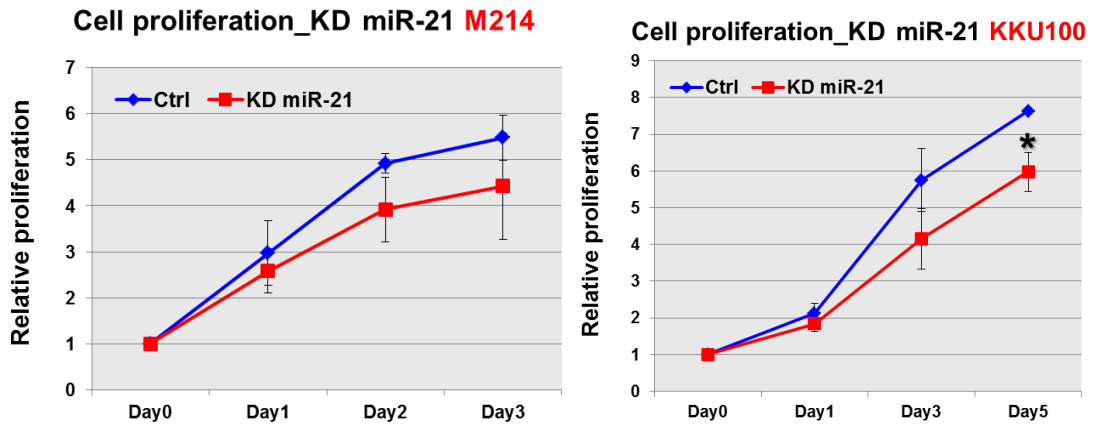


Fig 13.5 Decrease in cell proliferation in transient knock down CCA cell lines, KKU-M214 and KKU-100

13.5 Knocking down miR-21 in CCA cells elevated the levels of predicted target mRNAs

After 24 hr transient knocking-down of miR-21 in 2 CCA cell lines, KKU-M214 and KKU-100, the mRNA level of target genes was determined. Target genes were previously found to be downregulated in CCA tissues (unpublished data) were selected and predicted by MiRanda software. Highly changed target mRNAs, STK40, KLHL15, GPR64, ZTBT47 and CYBD1, were found in both cell lines. Moderately changed target mRNAs, FBX2, FNIP1 and SOX7, were found in both cell lines. CD44 was increased only in M214 (Fig. 13.6).

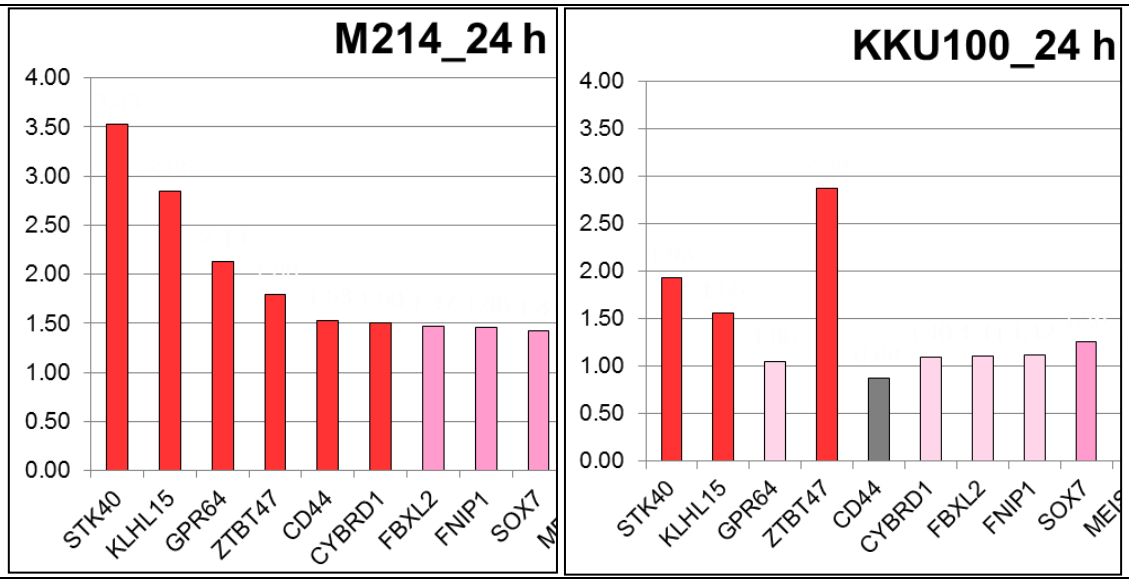


Fig. 13.6 Altered expression of target mRNAs upon 24-hr miR-21 knocking down in KKU-M124 and KKU-100 cell lines

13.6 Identification of potential direct targets of miR-21 by mRNA microarray

It is known that animal miRNAs regulate gene expression by inhibiting translation and/or by inducing degradation of target. In our study, most modulated genes on in the mRNA differential expression profiles changed by less than two-fold may still be miRNA targets; we defined differentially expressed genes as no less than 1.00-fold change. Candidate up-regulated mRNAs upon knocking down was analysed for miR-21 direct targets using several algorithms including miBridge (licensing software) combining with freely published prediction softwares that predict at 3'UTR seed region including TargetScan, PicTar and miRTarbase. Top 24 candidates were listed in Table 13.1.

Table 3.1 Regulated mRNAs in CCA cells after miR-21 knockdown determined by Affymatrix array

Item No.	Gene Name	Description	Fold change		Prediction program			
			KKU-100	KKU-M214	miBridge	TargetScan	PicTar	miRTarBase
1	ANKRD46	Ankyrin repeat domain 46	1.22	1.28	✓			
2	RECK	Reversion-inducing-cysteine-rich protein with kazal motifs	1.20	1.01		✓	✓	
3	CDC25A	Cell division cycle 25 homolog A (*S. pombe*)	1.15	1.07				✓
4	YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (S. cerevisiae)	1.10	1.15		✓	✓	
4	FAM63B	Family with sequence similarity 63, member B	1.12	1.13		✓		
5	C17orf39	Chromosome 17 open reading frame 39	1.10	1.07		✓	✓	
6	BCL2	B-cell CLL/lymphoma 2	1.10	1.04				✓
7	GLT8D3	Glycosyltransferase 8 domain containing 3	1.09	1.08		✓		
8	TPRG1L	Tumor protein p63 regulated 1-like	1.07	1.06	✓			
9	SOX6	SRY (sex determining region Y)-box 6	1.07	1.02	✓		✓	
10	SNTB2	Syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)	1.06	1.02		✓		
11	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	1.06	1.04		✓	✓	
12	TNRC6B	Trinucleotide repeat containing 6B	1.05	1.05	✓	✓	✓	
13	PCBP2	Poly(rC) binding protein 2	1.05	1.08		✓	✓	
14	ZCCHC3	Zinc finger, CCHC domain containing 3	1.05	1.05		✓	✓	
15	KLF9	Kruppel-like factor 9	1.05	1.04			✓	
16	MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	1.05	1.01				✓
17	GPR180	G protein-coupled receptor 180	1.04	1.02	✓			
18	NCAPG	Non-SMC condensin I complex, subunit G	1.02	1.00				✓
19	APIP	APAF1 interacting protein	1.02	1.01	✓			✓
20	SH3GLB1	SH3-domain GRB2-like endophilin B1	1.01	1.01	✓			
21	MPRIP	Myosin phosphatase Rho interacting protein	1.01	1.04		✓	✓	
22	PTEN	Phosphatase and tensin homolog	1.01	1.05	✓			✓
23	PCBP1	Poly(rC) binding protein 1	1.01	1.01				✓
24	TIMP3	TIMP metallopeptidase inhibitor 3	1.00	1.00		✓	✓	

13.7 Verification of miR-21 targets by qRT-PCR

After 48 hr transient knocking-down of miR-21 in 2 CCA cell lines, KKU-M214 and KKU-100, the mRNA level of ANKRD46, DDAH1, RECK, CDC25A, YOD1, GLT8D3, TPRG1L, PDCD4, APAF1, TRPC5, GPR180, TNRC6B, SH3GLB1 and SOX6 was verified by qRT-PCR. Increase in the mRNA level in both cell lines was observed for ANKRD46, DDAH1, RECK, CDC25A, YOD1, GLT8D3, TPRG1L and PDCD4 expression.

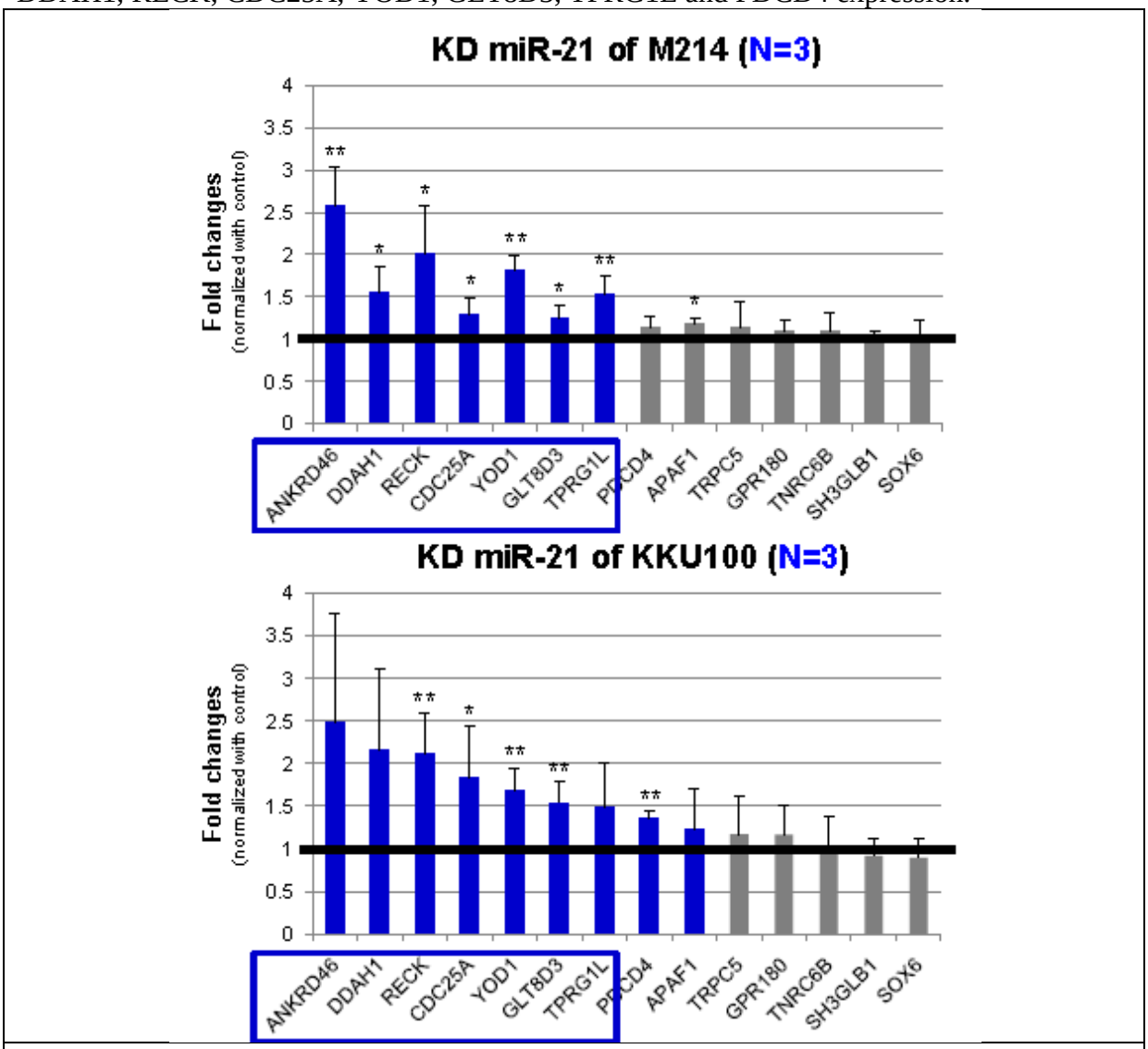


Fig. 13.7 Verification of miR-21 targets using qRT-PCR

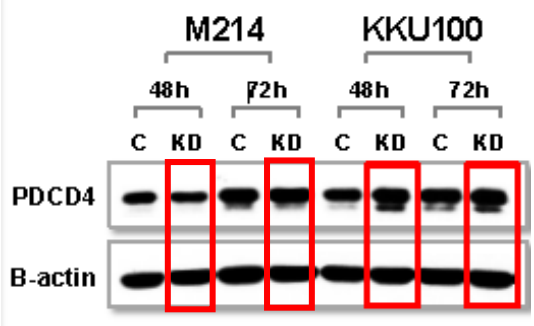


Fig. 13.8 Western blotting of PDCD4 protein levels in miR-21 knockdown cell lines

14. สรุปและวิจารณ์ผลการทดลอง (Summary and Discussion)

miR-21 is a key molecule in a wide range of cancers, and identifying its functional role in CCA has possibly biological implications. In CCA model, it indicates that miR-21 upregulation in early stage of carcinogenesis may play important role in the development of CCA. From animal to human study, the expression of miR-21 was also increased in tumor tissues of CCA patients. The expression of miR-21 was inversely correlated PDCD4 (cell death protein), the miR-21 targets, suggesting that miR-21 may regulate tumor cell growth via suppressing such molecules. We show here that knockdown of miR-21 suppresses cell proliferation of KKU-M214 and KKU-100 cell lines. Besides, knockdown of miR-21 increases PDCD4 mRNA and protein level. Therefore, our study strongly conclude that PDCD4 was down-regulated in CCA may strongly be mediated by miR-21 function. Regulatory network of miR-21 in CCA cells is also discovered from this study. New entries of miR-21 targets were analyzed by several methods.

Basically, many miRNAs with their 3'-end interaction sites in the 5'-UTRs turn out to simultaneously contain 5'-end interaction sites in the 3'-UTRs. Based on these finding, Lee and co-workers demonstrate combinatory interactions between a single miRNA and both end regions of an mRNA using model systems (Lee et al. 2009). They further show that genes exhibiting large-scale protein changes due to miRNA overexpression or deletion contain both UTR interaction sites predicted. They provide the predicted targets of this new miRNA target class, miBridge, as an efficient way to screen potential targets, especially for nonconserved miRNAs, since the target search space is reduced by an order of magnitude compared with the 39-UTR alone. Using miBridge (licensing software) combining with freely published prediction softwares that predict at 3'UTR seed region including TargetScan, PicTar and miRTarbase, we discover several directed targets of miR-21 including ANKRD46, DDAH1, RECK, CDC25A, YOD1, GLT8D3, TPRG1L, PDCD4, APAF1, TRPC5, GPR180, TNRC6B, SH3GLB1 and SOX6 that are upregulated upon knock down of miR-21 in CCA cells. Indirect downstream effectors of miR-21 are also investigated including STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1, SOX7 that play roles in cell differentiation are elevated upon miR-21 knockdown. Indeed, those miR-21 targets need to be further determining the clinical significance and the role in CCA, and it may provide the potential targets for CCA chemoprevention and treatment in the future.

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16. ภาคผนวก

16.1 การนำเสนอผลงานทางวิชาการจำนวน 2 ครั้ง

16.1.1 [Poster] **Namwat N**, Chusorn P, Loilome W, Wongkham S, Techasen A, Puapairoj A, Khuntikeo N, Yongvanit P. MiR-21 overexpression controls tumor cell growth and metastasis in cholangiocarcinoma of northeastern Thailand. New trends in health care ประชุมวิชาการประจำปี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ครั้งที่ 27 ประจำปี 2554 4-6 ตุลาคม 2554 Precongress : 1-3 ตุลาคม 2554 ณ ห้องบรรยาย คณะแพทยศาสตร์ (ได้รางวัลที่ 1 ประเภทบุคลากร)

16.1.2 [Oral] **Namwat N**, Chusorn P, Loilome W, Lee I,Techasen A, Subimerb C, Chan-On W, Ong CK, Pairojkul C, Khuntikeo N, Teh BT, Yongvanit P. MicroRNA-21 in intrahepatic cholangiocarcinoma and functional analysis. The First Symposium of

Specific Health Problem in Greater Mekong Sub-region (SHeP-GMS) Health Cluster, The National Research University Project. 11 May 2012 Faculty of Medicine, Khon Kaen University, Thailand.

16.2 ผลงานตีพิมพ์

16.2.1 Chusorn P, Namwat N, Loilome W, Dechakhumphu S, Yongvanit P. Overexpression of miR-21 controls the growth of *Opisthorchis viverrini*-associated cholangiocarcinoma mediated through PDCD4. (Manuscript in preparation)