PROTUMORIGENIC EFFECTS OF MIXED LINEAGE KINASE DOMAIN-LIKE PROTEIN IN CHOLANGIOCARCINOMA CELLS

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มะเร็งท่อน้ำดี เป็นมะเร็งที่เกิดจากเซลล์เยื่อบุผนังของท่อทางเดินน้ำดี โดยประเทศไทยพบอุบัติการณ์ของมะเร็งท่อน้ำดีสูงที่สุดในโลก โดยเฉพาะในแถบภาคตะวันออกเฉียงเหนือ มะเร็งท่อน้ำดีมีอัตราการเสียชีวิตที่สูง โดยอัตราการรอดชีวิตในระยะเวลา 5 ปี ของคนไข้ในระยะท้ายต่ำเพียง 5-10% เท่านั้น เนื่องจากการวินิจฉัยในระยะเริ่มต้นยังทำได้ยากและยังคงขาดวิธีการรักษาที่มีประสิทธิภาพ อีกทั้งยังขาดความรู้ความเข้าใจเกี่ยวกับกลไกการเจริญพัฒนาของมะเร็งท่อน้ำดี ดังนั้นการศึกษากลไกการเจริญพัฒนาของมะเร็งท่อน้ำดีจึงมีความจำเป็นเร่งด่วน เพื่อใช้พัฒนาเป็นเป้าหมายใหม่ในการรักษามะเร็งท่อน้ำดีให้มีความจำเพาะและมีประสิทธิภาพมากขึ้น โปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ เป็นโปรตีนสำคัญในวิถีการตายของเซลล์แบบใหม่ เรียกว่า เนครอปโทซิส การศึกษาก่อนหน้ารายงานว่าโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์มีบทบาทใหม่ที่ไม่เกี่ยวข้องกับวิถีการตายของเซลล์แบบเนครอปโทซิสและอาจมีบทบาทในการส่งเสริมการเจริญพัฒนาของมะเร็ง แต่อย่างไรก็ตามในปัจจุบันยังไม่มีการศึกษาบทบาทของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ในมะเร็งท่อน้ำดี จากการศึกษาก่อนหน้าในชิ้นเนื้อของคนไข้มะเร็งท่อน้ำดี พบว่าโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์มีการแสดงออกที่สูงขึ้นอย่างมีนัยสำคัญ นอกจากนั้นพบว่าคนไข้ที่มีระดับการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ที่สูงมีความสัมพันธ์กับอัตราการรอดชีวิตและการพยากรณ์โรคที่ต่ำ และอัตราการกลับเป็นซ้ำที่สูงขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับคนไข้ที่มีระดับการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ที่ต่ำ นอกจากนั้นยังพบว่าคนไข้ที่มีระดับการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์สูงมีความสัมพันธ์ในทางบวกกับกลุ่มเซลล์ในระบบภูมิคุ้มกันที่ทำหน้าที่กดภูมิคุ้มกัน ดังนั้นจากผลข้างต้นแสดงให้เห็นว่าโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์อาจจะมีบทบาทในการส่งเสริมการเจริญและพัฒนาของมะเร็งท่อน้ำดีและมีบทบาทสำคัญในมะเร็งท่อน้ำดี ดังนั้นในงานวิจัยนี้ผู้วิจัยจึงสนใจศึกษาบทบาทของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ในมะเร็งท่อน้ำดี โดยคณะผู้วิจัยได้ทำการศึกษาเพิ่มเติมโดยการวิเคราะห์หาความสัมพันธ์ระหว่างโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์กับกลุ่มยีนเอกลักษณ์ที่เกี่ยวข้องกับคุณลักษณะของเซลล์มะเร็ง ได้แก่ การแบ่งตัวเพิ่มจำนวน กระบวนการเปลี่ยนแปลงรูปร่างของเซลล์จากเซลล์เยื่อบุผิวเป็นเซลล์ที่มีรูปร่างแบบกระสวย การเคลื่อนที่และการบุกรุกของเซลล์ โดยใช้ฐานข้อมูลสาธารณะ GEO database การศึกษาพบว่าโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์มีความสัมพันธ์เชิงบวกกับกลุ่มยีนเอกลักษณ์ดังกล่าวอย่างมีนัยสำคัญทางสถิติ ดังนั้นจึงนำไปสู่การตั้งสมมติฐานว่า โปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์มีบทบาทในการส่งเสริมการเจริญและพัฒนาของเซลล์มะเร็งท่อน้ำดี โดยการส่งเสริมการแบ่งตัวเพิ่มจำนวน การกระตุ้นให้เกิดกระบวนการเปลี่ยนแปลงรูปร่างของเซลล์จากเซลล์เยื่อบุผิวเป็นเซลล์ที่มีรูปร่างแบบกระสวย การส่งเสริมให้มีการเคลื่อนที่ และการบุกรุกของเซลล์มะเร็ง คณะผู้วิจัยพบว่าเมื่อยับยั้งการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์โดยใช้เทคนิค short hairpin RNA (shRNA) ในเซลล์ไลน์มะเร็งท่อน้ำดีจำนวน 2 ชนิด ได้แก่ KKU213 และ HuCCT-1 ส่งผลให้ความสามารถในการแบ่งตัวเพิ่มจำนวนลดลงอย่างมีนัยสำคัญทางสถิติ การศึกษาเพิ่มเติมพบว่าเซลล์ที่ไม่มีการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์มีการเพิ่มจำนวนเซลล์ในระยะ S phase พบการแสดงออกที่ลดลงของยีนที่ส่งเสริมการเกิดการเปลี่ยนแปลงรูปร่างของเซลล์จากเซลล์เยื่อบุผิวเป็นเซลล์ที่มีรูปร่างแบบกระสวย การเคลื่อนที่และการบุกรุกของเซลล์ลดลงอย่างมีนัยสำคัญทางสถิติในเซลล์ที่ไม่มีการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์เมื่อเปรียบเทียบกับเซลล์ควบคุมที่มีการแสดงออกของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ในระดับปกติ ดังนั้นงานวิจัยนี้จึงเป็นงานวิจัยแรกที่รายงานเกี่ยวกับบทบาทใหม่ของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์ในการส่งเสริมการเจริญและพัฒนาของเซลล์มะเร็งท่อน้ำดี แต่อย่างไรก็ตามยังต้องมีการศึกษาเพิ่มเติมเกี่ยวกับกลไกการทำงานของโปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์และการศึกษาบทบาทในสัตว์ทดลอง ดังนั้นในอนาคตหากมีการศึกษาต่อยอด โปรตีนมิกซ์ลินเนจไคเนสโดเมนไลค์น่าจะสามารถพัฒนาใช้เป็นเป้าหมายใหม่ในการพัฒนาวิธีการรักษามะเร็งท่อน้ำดีแบบมุ่งเป้า ที่มีประสิทธิภาพมากขึ้น

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Nattaya Duangthim : PROTUMORIGENIC EFFECTS OF MIXED LINEAGE KINASE DOMAIN-LIKE PROTEIN IN CHOLANGIOCARCINOMA CELLS. Advisor: Asst. Prof. SIRIPORN JITKAEW, Ph.D.

Cholangiocarcinoma (CCA), a heterogeneous malignancy of bile duct system, has the highest incidence in Thailand. CCA is one of the leading causes of death which has low 5-year survival rate from 5% to 10% in advanced stages due to the difficulty of diagnosis in early stage and lack of effective therapies. Therefore, understanding the molecular mechanisms and pathogenesis of CCA development and progression is urgently needs and will lead to the development of novel therapeutic targets and strategies. Mixed lineage kinase domain-like (MLKL) was identified as a key effector in a novel regulated necrosis or necroptosis pathway. Recently studies have reported that MLKL may contribute to cancer development and progression which is independent of necroptosis function, however the functional roles of MLKL in cancer need further investigation. We therefore were particularly interested to explore the role of MLKL in CCA. Our preliminary studies found that MLKL expression was significantly increased in human CCA primary tissues. More importantly, high MLKL expression was significantly associated with both shorter disease-free survival (DFS) and overall survival (OS) and were positively correlated with an unfavorable immune signature and immunosuppression. Therefore, these results suggested that MLKL might contribute to CCA development and progression. Moreover, we analyzed the correlation of MLKL mRNA expression and several genes signature-associated with hallmarks of cancer from public datasets. MLKL was positively correlated with genes-associated with proliferation, epithelial-mesenchymal transition (EMT), migration and invasion in CCA. We therefore hypothesized that MLKL has pro-tumorigenic roles through promoting proliferation, epithelial–mesenchymal transition (EMT), migration and invasion in CCA cells. To study the role of MLKL in CCA cells, we made use of short-hairpin RNA (shRNA) to silence MLKL expression in two representative CCA cell lines including KKU213 and HuCCT-1 cells. Interestingly, our present study demonstrated that cell proliferation analyzed by MTT assay and colony formation is significantly decreased in MLKL knockdown cells compared to control cells. Cell cycle analysis showed that percentage of cells in S phase is higher in MLKL knockdown cells than control cells which suggest that MLKL might contribute to cell cycle regulation. In addition, following treatment with TNF-α, the expression of vimentin, one of genes in EMT process is significantly lower expressed in MLKL knockdown cells compared to control cells. In line, both migration and invasion which were analyzed by wound healing and transwell migration assay are significantly decreased in MLKL knockdown cells compared to control cells. Taken together, our finding is the first study to reveal that MLKL might have pro-tumorigenic role by promoting cell proliferation, EMT, migration and invasion in CCA cells. Further studies including mechanistic studies of how MLKL promote CCA development and progression and validation in animal model are needed. Our preliminary *in vitro* results therefore suggest that MLKL might be a promising therapeutic target for CCA targeted therapy.

Field of Study:	Clinical Biochemistry and Molecular Medicine	Student's Signature	ณัฐธยาน์ ดวงธิม
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Nattaya Duangthim

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CHAPTER I

INTRODUCTION

1.1 BACKGROUND AND RATIONALE

Cholangiocarcinoma (CCA) is a heterogeneous malignancy originated from normal bile ducts. CCA is the second most common form of primary liver cancer and is well known to have the highest incidence in Thailand especially in North-East provinces (1). Nowadays, CCA has high mortality rate, and 5-year survival rate is only 5-10% for the patients with advanced stages because of the difficulty of early diagnosis and lack of effective therapy (2). Therefore, understanding the molecular pathogenesis of CCA will lead to development of the potential prognostic biomarkers and novel therapeutic target and strategies.

Necroptosis is a regulated form of necrosis and a caspase-independent cell death (3). There are 3 key components in this pathway consisting of receptor-interacting protein kinase 1 (RIPK1), receptor-interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL). Accumulating studies have reported that necroptosis and its key regulators contribute to many pathologies and diseases including cancer (4). Nowadays, many studies have found that key necroptotic effectors might have functions independent of necroptosis activation. In this study, we were particularly interested to explore the role of RIPK3 and MLKL in CCA because the roles of RIPK3 and MLKL in CCA are still unknown while the functions of RIPK1 are more studied and have been shown to contribute to many biological processes in other solid cancers (4). In order to understand more about RIPK3 and MLKL in CCA, we analyzed RNA sequencing data from the public database at The Cancer Genome Atlas (TCGA) National Cancer Institute. They have performed RNA sequencing obtained from 36 cholangiocarcinoma samples and 9 normal bile ducts. We found that RIPK3 and MLKL were highly expressed in human cholangiocarcinoma compared to the normal bile duct tissues (5). In addition, we have performed immunohistochemistry analysis

to examine RIPK3 and MLKL protein expression in 89 paraffin-embedded CCA tissue samples. Similar to mRNA expression, both RIPK3 and MLKL expression in CCA tissues were differentially expressed in CCA primary tissues. However, only MLKL, but not RIPK3 was significantly higher expressed in tumor tissues than the adjacent tissues (6). Therefore, these results suggested that upregulation of MLKL in CCA primary tissues might play a role in CCA.

MLKL is a pseudokinase protein that was identified in 2012 as a key effector and downstream target of RIPK3 in a novel regulated necrosis or necroptosis pathway (7). However, recent data suggest that MLKL has alternative functions independent of necroptosis. For example, depletion of MLKL enhances TRAIL-induced cell death which is independent of necroptosis function (8). In addition, MLKL was highly expressed in hepatic stellate cells (HSCs) which contributes to hepatic fibrosis and that MLKL inhibition delays wound healing and vimentin expression in HSCs (9). A recent study in nasopharyngeal carcinoma (NPC) cells has demonstrated that the depletion of MLKL expression inhibits invasion and significantly increases epithelial markers (E-Cadherin) and decreases mesenchymal markers (Vimentin, N-Cadherin, Zeb1) of radioresistant NPC cells (10). However, the functional roles and a deeper mechanistic understanding of MLKL in cancer need further investigation. Although the role of MLKL in cancer is still unclear, many studies have found that low MLKL expression was significantly associated with both shorter disease-free survival (DFS) and overall survival (OS) in patients in several types of cancers such as early-stage resected pancreatic cancer (11), ovarian cancer (12), cervical cancer (13), gastric cancer (14) and colon cancer (15). However, the impact of MLKL expression on patient survival rates are still unknown in CCA. Our preliminary study revealed that high MLKL expression was significantly associated with both shorter DFS and OS. However, this result is contrast to the correlation between pMLKL, a specific marker of necroptosis activation and survival rate of CCA patients in which high pMLKL expression showed a trend of longer DFS

and OS (6). Therefore, these results indicated that MLKL might contribute to CCA through a non-necroptotic function.

CCA is associated with chronic inflammation and immunosuppression which are known to influence the biological behavior of tumor and therapeutic outcomes. The analysis of the correlation between MLKL and tumor-infiltrating immune cells including $CD8^+$ T cells, an anti-tumor leukocyte and tumor-associated macrophages (TAMs), a tumor promoting leukocyte demonstrated that high MLKL expression was negatively correlated with $CD8^+$ T cells but positively correlated with TAMs which is in contrast to the results of pMLKL (6). Therefore, these results suggested that MLKL might be associated with pro-inflammatory responses in CCA tumor microenvironment. In this study, in order to further dissect the potential protumorigenic roles of MLKL in CCA, we have analyzed the correlation between MLKL mRNA expression and genes-associated with proliferation, epithelial-mesenchymal transition (EMT), migration and invasion from the Gene Expression Omnibus (GEO) database; GSE76297 (N = 91) and GSE107943 (N = 30). Our results demonstrated that MLKL was positively correlated with several genes-associated with hallmarks of cancer mentioned above. This led us to hypothesized that MLKL might promote proliferation, EMT, migration and invasion in CCA cells.

Therefore, in our present study, we aimed to investigate the protumorigenic roles of MLKL in CCA, inhibition of MLKL expression from CCA cells that express a functional MLKL was used to resolve these roles. RNA interference-short hairpin RNA (shRNA) was used to generate MLKL knockdown CCA cell models and these cell models were used to evaluate the protumorigenic roles of MLKL in CCA cells. To the best of our knowledge, this is the first research to reveal the protumorigenic roles of MLKL in CCA. Finally, better understanding the protumorigenic roles of MLKL in CCA will lead to development of a novel therapeutic target for CCA treatment.

1.2 RESEARCH QUESTION

Does MLKL have protumorigenic roles in cholangiocarcinoma cells?

1.3 HYPOTHESIS

MLKL promotes proliferation, EMT, migration and invasion in cholangiocarcinoma cells.

1.4 OBJECTIVES (AIMS)

1.4.1 To generate MLKL knockdown CCA cell models

1.4.2 To investigate the roles of MLKL on proliferation, EMT, migration and invasion in CCA cells *in vitro*

1.5 CONCEPTUAL FRAMEWORK

1.6 SCOPE OF RESEARCH

Two representative CCA cell lines which have high MLKL expression, invasive phenotypes and high RIPK3 were used as an *in vitro* model. Next, loss of function genetic tools called RNA interference-short hairpin RNA (shRNA) was used to generate MLKL knockdown CCA cell models for investigation the protomorigenic roles of MLKL in CCA *in vitro*. After that, MTT and colony formation assays were used to assess the effect of MLKL knockdown on CCA proliferation. qRT-PCR was used to determine the expression level of epithelial and mesenchymal cell markers in MLKL knockdown CCA cells compared to non-targeting control CCA cells. Finally, the effect of MLKL knockdown on CCA migration and invasion were determined by using Wound healing assay and Transwell matrigel invasion assay compared to non-targeting control CCA cells, respectively. In this study, all experiments were carried out in three independent experiments.

1.7 BENEFIT AND APPLICATION

In this study, we were the first study to reveal the protumorigenic roles of MLKL in cholangiocarcinoma (CCA) by using MLKL knockdown CCA cell as a model. Better understanding the protumorigenic roles of MLKL in CCA will lead to the development of a novel therapeutic target for CCA treatment. However, the underlying mechanism how does MLKL promote CCA development and progression should be further investigated.

CHAPTER II

LITERATURE REVIEW

2.1 CHOLANGIOCARCINOMA

Cholangiocarcinoma (CCA) is a heterogeneous malignancy that originated from normal bile ducts. CCA is the second most common form of primary liver cancer after hepatocellular carcinoma (HCC). By anatomical classification, CCA is divided into 3 types which are intrahepatic (iCCA), perihilar (pCCA) and extrahepatic cholangiocarcinoma (dCCA) (Figure 1) (16). Although CCA is well known to have the highest incidence in Thailand especially in North-East provinces, the incidence rate has progressively increased worldwide. There are many risk factors associated with CCA development such as primary sclerosing cholangitis (PSC), choledochal cystic disease, hepatitis B/C infection, hepatolithiasis, inflammatory bowel disease (IBD), alcohol consumption and tobacco use. However, the most common risk factors of CCA in Thailand is *Opisthorchis viverrini* infection (17, 18).

Nowadays, there are many methods for CCA treatment such as surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy. Surgery is only possible curative treatment for CCA. Unfortunately, no specific symptoms are observed during early stage and no specific early-stage biomarkers have been identified, hence CCA is usually detected in the late stage in which surgery resection cannot be done. Chemotherapy and radiotherapy become the next treatment options for CCA treatment. However, most of CCA patients are developed chemoresistance and have severe side effects. Hence, curative treatments for CCA remain disappointing (19). Therefore, it is so urgent to identify the early diagnostic/prognostic biomarkers and develop novel therapeutic targets for CCA. A better understanding of CCA molecular pathogenesis will lead to the development of a potential early diagnostic/prognostic biomarkers and novel therapeutic target and strategies.

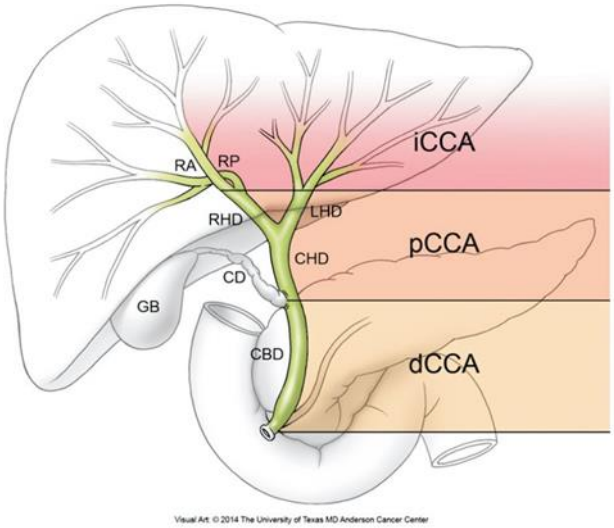

Figure 1 The types of cholangiocarcinoma (CCA) based on tumor location (16)

2.1.1 MOLECULAR PATHOGENESIS OF CHOLANGIOCARCINOMA

Although the above risk factors associate with several diseases, pathological characteristics in almost all of them are associated with chronic inflammation (18). Chronic Inflammation is well known as the key factors in cholangiocarcinogenesis by effecting on several intracellular pathways that contribute to CCA development and progression. Since chronic inflammation causes cholangiocytes to remain expose to the inflammatory factors such as Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Transforming Growth Factor-β (TGF-β), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor and Cyclooxygenase-2 (COX-2), resulting in mutations of tumor suppressor genes, proto-oncogenes, and DNA mismatch-repair (MMR) genes (18, 20-22).

Many studies reported that IL-6 affects several intracellular pathways that contribute to CCA development and progression. For example, IL-6 can downregulate specific microRNAs resulting in increased DNMT1 which is an enzyme to methylate cytosine resulting in genetic mutation such as suppression of the tumor suppressor genes expression (e.g. p16INK4a and Rassf1a), and finally lead to cholangiocarcinogenesis (20). Moreover, IL-6 also increases progranulin expressions,

leading to Akt pathway activation which regulates cell growth, migration and angiogenesis (23). In addition, in case of *O. viverrini* infection, this liver fluke can secrete Ov-GRN-1 that promotes angiogenesis, suppresses apoptosis, and promotes tumor invasion (Figure 2) (24). TNF-**α**, a proinflammatory cytokine can upregulate the expression of Activation-Induced Cytidine Deaminase (AID), an enzyme converting cytosine to uracil which finally lead to genetic mutation such as suppressing tumor suppressor gene, p53 or activation proto-oncogene, MYC (21). In case of *O. viverrini* infection, this fluke can secrete inducible nitric oxide synthase (iNOS) which regulates COX-2 activity and increases nitric oxide (NO) production, resulting in oxidative DNA damage by affecting DNA repair mechanisms (Figure 3) (25, 26).

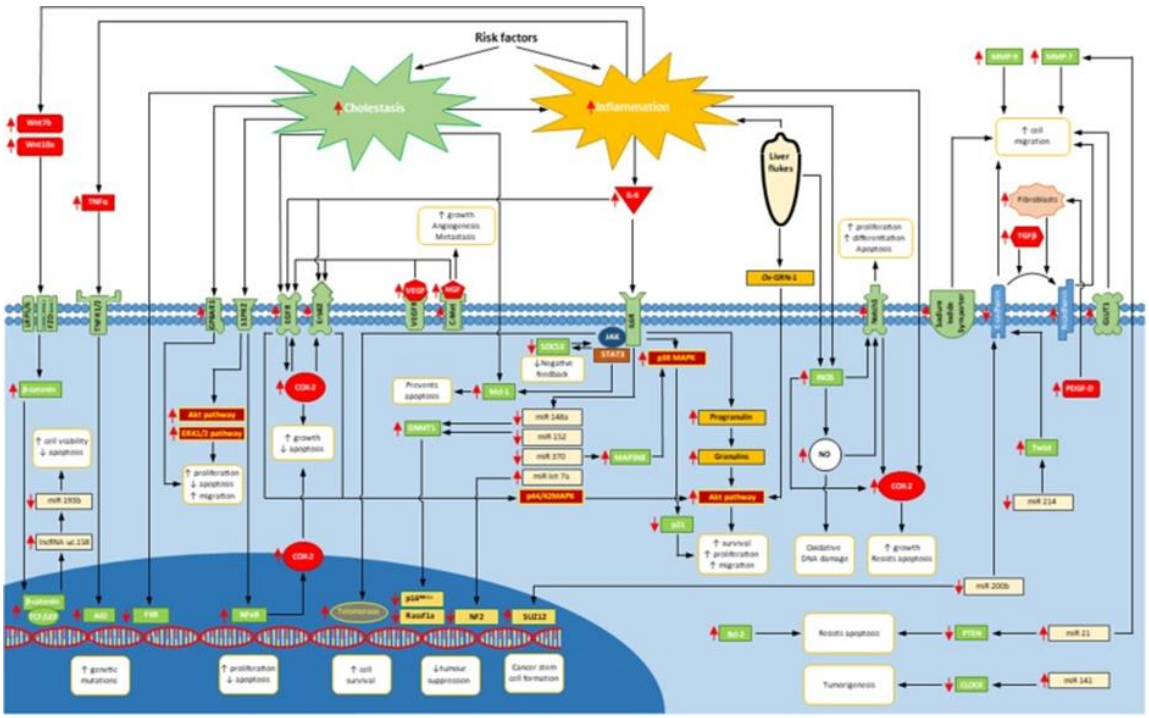

Figure 2 Chronic inflammation effects on several intracellular signaling pathways, resulting in cholangiocarcinogenesis (18)

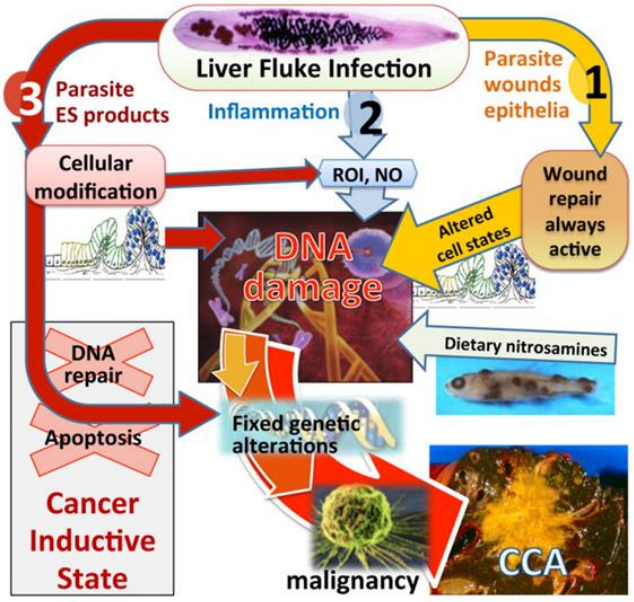

Figure 3 Pathogenesis pathways of liver fluke-induced cholangiocarcinoma (CCA)

(27)

2.2 NECROPTOSIS

Necroptosis is a regulated form of necrosis and a caspase-independent cell death. There are 3 key components in this pathway consisting of receptor-interacting protein kinase 1 (RIPK1), receptor-interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL). Necroptosis can be induced by many stimuli such as Toll-like receptor 3 (TLR3), TLR4, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), Interferon receptor, T cell receptor, Intracellular RNA- and DNA-sensing molecules but the most studied model of necroptosis is TNF signaling model (28, 29). When TNF-α binds to TNF receptor, complex I is formed which results in NF-kB activation and finally results in inflammation and cell survival. But when complex I is blocked by Smac-mimetic (IAPs antagonist), it leads to the formation of complex IIa and finally leads to apoptosis. When both complex I and complex IIa are blocked by Smac-mimetic and zVAD (pancaspase inhibitor). It leads to the recruitment of RIPK1 and RIPK3 to form the complex called necrosome. Next, RIPK3 phosphorylates MLKL at Threonine 357 and Serine 358 to activate MLKL. After that, activated MLKL forms

homotrimer and finally homotrimer MLKL will translocate to plasma membrane which causes plasma membrane rupture and releases damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 (HMGB-1), heat shock proteins (HSPs), F-actin, DNA, RNA and extracellular ATP which can trigger immune responses and inflammation and finally leads to necroptosis (Figure 4) (28).

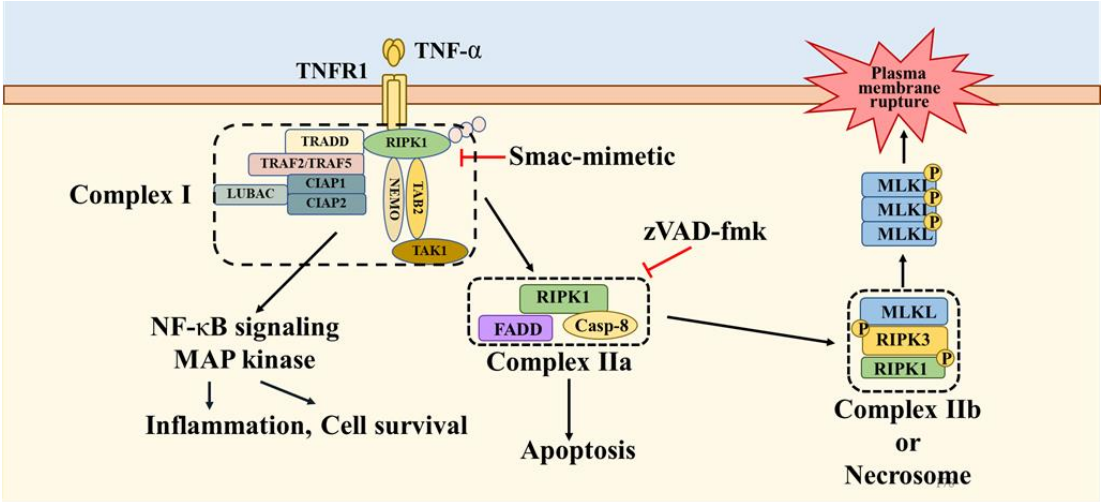

Figure 4 The pathway of necroptosis; TNF signaling model, modified from (28)

2.3 RECEPTOR-INTERACTING PROTEIN KINASE 3 (RIPK3)

RIPK3 is a serine/threonine kinase in RIP kinase family that was identified as a key effector of necroptosis pathway in 2009 (30). The human RIPK3 gene is located on chromosome 14 and its mRNA encodes 518 amino acids (31). RIPK3 has 2 main structural components, one is N terminus which is an active kinase domain that is conserved in other RIP kinases, and another is C terminus which is a protein-protein interaction domain, and this part is unique in each RIP family member. Such as RIPK3, C-terminus is called the RIP homotypic interaction motif (RHIM domain), which interacts with RIPK1 in necroptosis pathway (31).

2.4 MIXED LINEAGE KINASE DOMAIN-LIKE (MLKL)

Mixed lineage kinase domain-like (MLKL) is a pseudokinase family that was identified as a key effector and downstream target of RIPK3 in necroptosis pathway in

2012 (7, 32). The human MLKL gene is located on chromosome 16. In 2012, two distinct MLKL isoforms which are generated by alternative splicing were reported in human colon adenocarcinoma HT-29 cells (32). Isoform 1 (MLKL1) serves as long MLKL isoform which encodes the full-length 471 amino acids while isoform 2 (MLKL2) encodes 263 amino acids due to lack of exons 4-8. The structure of MLKL1 consists of N-terminal four-helix bundle (4HB) domain and C-terminal pseudokinase domain (PsKD) which are connected by two-helix linker called brace helices whereas MLKL2 has the same N-terminus as MLKL1 but lacking much of the C-terminal pseudokinase and two RIPK3 phosphorylation site on PsKD activation loop (Figure 5). Nowadays, the 4HB domain was identified as the death effector domain within MLKL because it is responsible for oligomerization and plasma membrane rupture in necroptosis pathway. PsKD functions serves as a molecular switching, where RIPK3 phosphorylates at Threonine 357 and Serine 358 within PsKD activation loop of MLKL, resulting in PsKD conformational change and unleash the 4HB domain to oligomerize and associate with plasma membrane and finally lead to necroptosis.

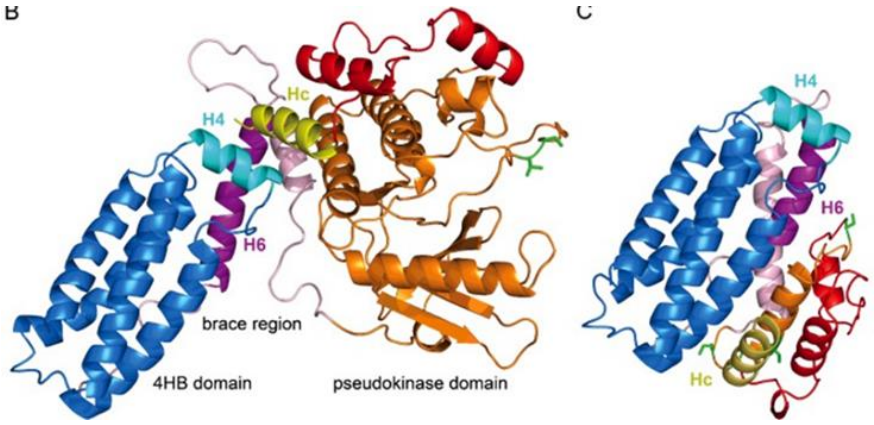

Figure 5 The crystal structure of human MLKL isoform1 (Left) and isoform2 (Right)

(33)

2.4.1 THE TISSUE DISTRIBUTION OF MLKL

Based on RNA sequencing datasets (HPA, GTEx and FANTOM5) collected from 55 tissue types and 6 blood cell types showed that MLKL was expressed in all tissues especially vagina, spleen, appendix, monocyte and bone marrow (Figure 6) (34).

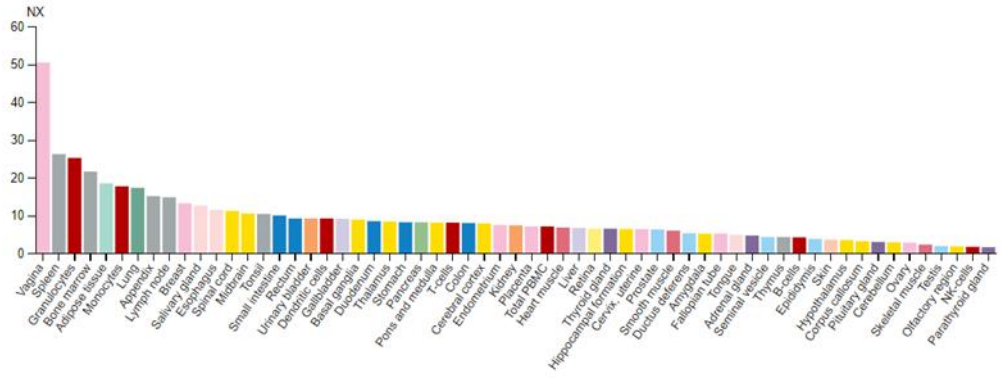

Figure 6 The tissue distribution of MLKL

(34)

2.5 NECROPTOSIS AND ITS CONTRIBUTION TO PATHOGENESIS OF HUMAN DISEASES

2.5.1 NECROPTOSIS AND NEURODEGENERATIVE DISEASES

Several studies reported that necroptosis caused pathogenesis of many neurodegenerative diseases such as Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Multiple sclerosis (MS), Spinal cord injury, Traumatic brain injury (TBI) and Alzheimer's disease (AD) (35).

The pathological characteristics of PD are dysfunctions in mitochondrial dynamics and metabolism. In 2016, Ito Y, *et al*. found that in models of Parkinson's disease (PD), mitochondrial morphological alterations and mitochondrial dysfunction in dopaminergic neurons was reduced after inhibition of RIPK1 (36). Hence, these results indicated that necroptosis acts as a potential therapeutic target for PD. In mouse ALS models, necroptosis pathway was increased in neural tissue (37). In addition, motor dysfunction in the SOD1G93A mouse model of ALS was delayed after inhibition of RIPK1 and RIPK3. These results suggested that necroptosis especially RIPK1 and RIPK3

play an important role in ALS pathogenesis. Moreover, the characteristic of multiple sclerosis (MS) is loss of oligodendrocytes, demyelination in the brain and spinal cord white matter and axonal degeneration. In 2015, Ofengeim D, *et al*. demonstrated that the activation form of key necroptotic proteins were markedly increased in human MS cortical lesions (38). In addition, oligodendrocytes in MS animal model were protected from necroptosis cell death after inhibition of RIPK1. Thus, these results suggested that necroptosis might associated with MS pathogenesis and RIPK1 might serve as potential therapeutic target for MS treatment.

2.5.2 NECROPTOSIS AND HEPATIC DISEASES

Many studies found that necroptosis involves in many hepatic diseases such as Nonalcoholic fatty liver disease (NAFLD), Autoimmune hepatitis and Nonalcoholic Steatohepatitis (NASH).

The pathological characteristics of non-alcoholic fatty liver disease (NAFLD) are hepatocyte cell death, inflammation and oxidative stress constitute. In 2015, Afonso MB, *et al*. demonstrated that increasing of circulating RIPK3 and phosphorylated MLKL have been observed in NAFLD patients (39). In addition, MCD diet-induced liver injury, steatosis, inflammation, fibrosis and oxidative stress were decreased after inhibition of RIPK3. Therefore, these results indicated that necroptosis mediated NAFLD progression and RIPK3 might serve as potential therapeutic target for NAFLD treatment. Moreover, in 2014, Luedde M, *et al*. reported that MLKL was upregulated in human autoimmune hepatitis (40). In addition, they found that MLKL mediates programmed hepatocellular necrosis independently of RIPK3 during hepatitis. And they found that in hepatic inflammation, IFN-γ induced MLKL expression through activation of the transcription factor STAT1. Altogether, these studies indicated MLKL was associated with the pathogenesis of hepatic diseases.

2.5.3 NECROPTOSIS AND CARDIOVASCULAR DISEASES

Several studies have reported that necroptotic proteins involve in many cardiovascular diseases such as heart failure, myocardial injury (MI), aortic aneurysm (AAA), ischemic neural injury and stroke.

In general, necrotic death of macrophages is well known to be present in atherosclerotic lesions. In 2013, Lin J, *et al*. found that after inhibition RIPK3 in macrophages, advanced atherosclerotic lesions and necrotic death of macrophages were reduced (41). Therefore, suggesting that macrophage necrosis in atherosclerosis development was mediated by RIPK3.

In addition, the pathological feature of abdominal aortic aneurysm (AAA) is lack of medial smooth muscle cell (SMC). In 2015, Wang Q, *et al*., reported that activation RIPK3 signaling in aneurysmal tissues leads to SMC necroptosis and promoting vascular inflammation, finally resulting in AAA development and progression (42). These results indicated that AAA progression might mediated by RIPK3 and RIPK3 might serve as potential therapeutic target for AAA treatment.

2.5.4 NECROPTOSIS IN CANCER

Nowadays, several studies reported that necroptosis and key necroptotic proteins have double-edge sword in cancer. As we all know, necroptosis initiates adaptive immune responses by releasing DAMPs into the tumor microenvironment for eliminating cancer. Nevertheless, necroptosis can also trigger inflammatory responses and promotes cancer development and progression.

2.5.4.1 NECROPTOSIS IN PROTECTING FROM TUMORIGENESIS

Dominique B, *et al*.,2016 studied the role of RIPK3 in colon cancer by using Wild-type (WT) and RIPK3-/- mice in which mice were injected with the carcinogen azoxymethane (AOM) and 2% Dextran sodium sulfate (DSS) to generate model of inflammation-driven colon cancer (43). They found that tumor growth was increased in the middle and distal part of the colon in RIPK3-/- mice (Figure 7; Left) and the

number of the larger tumor size was also increased (Figure 7; Middle). In addition, RIPK3-/- mice had a shorter in % survival rate when compared to the WT mice (Figure 7; Right). These results implied that RIPK3 plays an important role in suppressing colon cancer.

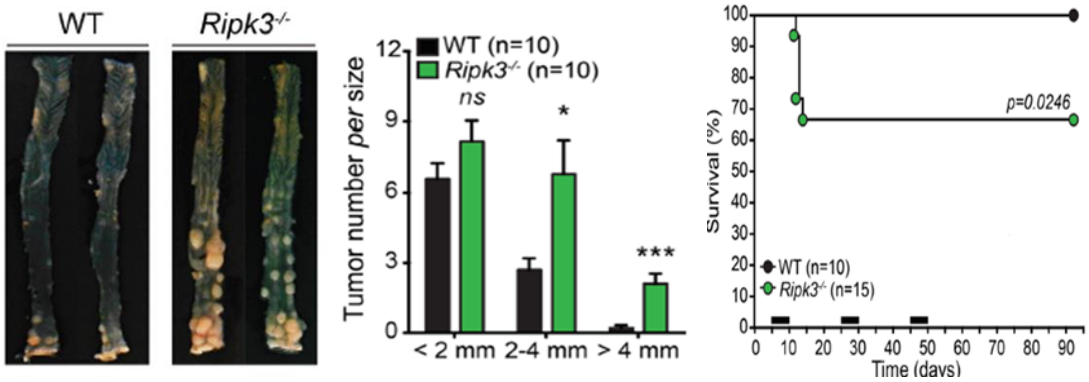

Figure 7 Necroptosis in protecting from tumorigenesis in WT and RIPK3-/- mice (43)

In addition, this study found that RIPK3-/- mice was highly increased colon inflammation (Figure 8; Left), thus real time qPCR was used to examine the expression of pro-inflammatory cytokines and chemokines in tumors of WT and RIPK3-/- mice. They found that the colon tumor of RIPK3-/- mice increased the transcriptional genes that encode cytokines such as TNF- α, IL-6, IL-1β, and IL-11 (Figure 8; Right). Therefore, these results suggested that RIPK3 has anti-inflammatory and anti-tumor functions in the intestine (43).

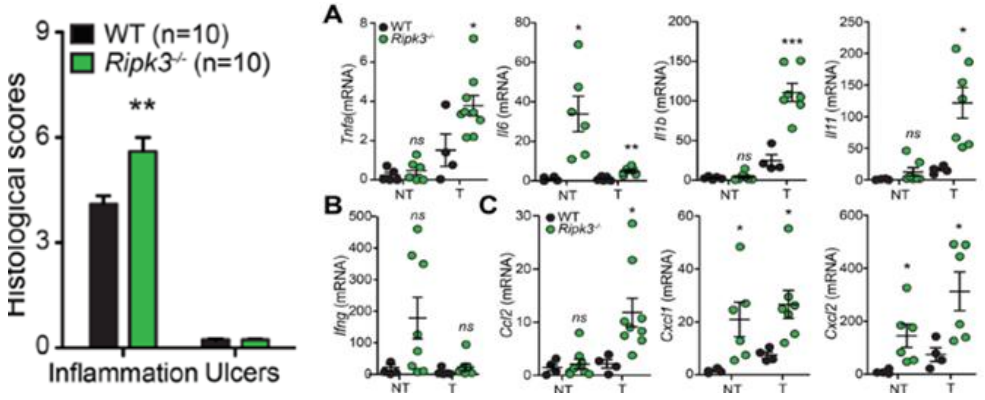

Figure 8 The result of real time qPCR showed the pro-inflammatory cytokines and chemokines expression in tumors of WT and RIPK3 knockout mice

(43)

Apart from the above study, Gautier S, *et al*., 2017 determined the expression levels of RIPK1, RIPK3 and MLKL in two major breast cancer-relevant databases including TCGA and molecular taxonomy of breast cancer international consortium (METABRIC) (44). They found that MLKL expression was decreased in tumor when compared with normal tissues (Figure 9). Moreover, they examined the correlation between necroptotic proteins and cellular immune cells in two independent breast cancer cohorts (TCGA and METABRIC dataset) using Spearman correlation test. They found that MLKL which represents in red color were strongly positive correlated with cellular immune system including myeloid and lymphoid cells (Figure 10). Hence, these results indicated that MLKL can eliminate breast cancer by stimulating anti-tumor immune response.

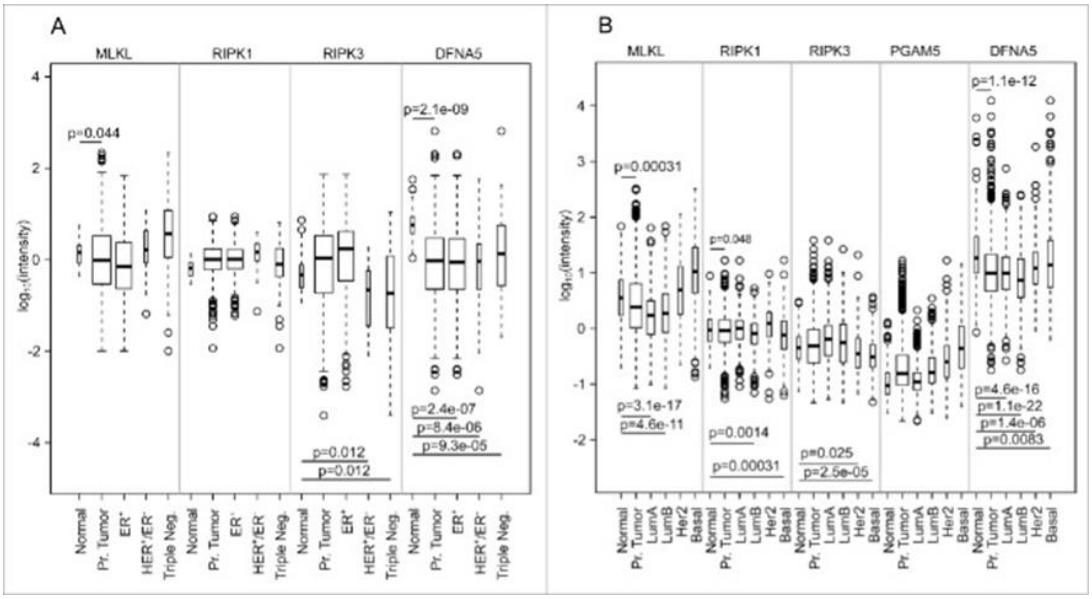

Figure 9 Expression of MLKL, RIPK1, RIPK3, PGAM5 and DFNA5 in breast cancer (A) TCGA data set (B) METABRIC data set

(44)

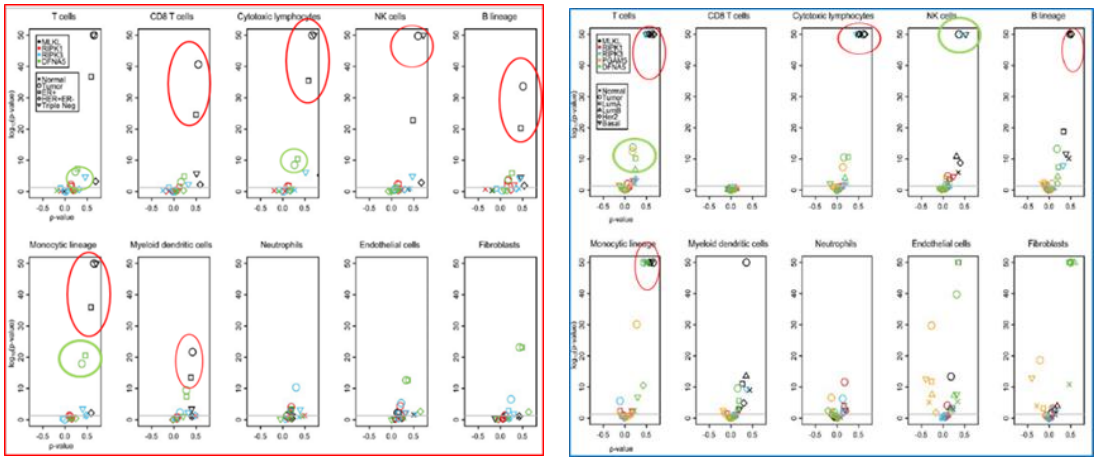

Figure 10 Volcano plots of Spearman correlation test between necroptotic proteins and immune cells (A) TCGA data set (B) METABRIC data set (44)

Moreover, Qun Z, *et al.,* 2019 studied the role of MLKL in colon cancer by using Wild-type (WT) and MLKL-/- mice which were injected the carcinogen azoxymethane (AOM) and 3% Dextran sodium sulfate (DSS) to generate model of inflammation-driven colon cancer (45). They found that at day 6 of DSS treatment, the body weight loss and clinical severity were increased in MLKL−/− mice but shorter colon lengths when compared with WT mice. Moreover, enlarged spleens and mesenteric lymph nodes (mLN), an inflammatory phenotype, were observed in DSS-fed MLKL−/− mice (Figure 11). These results suggested that MLKL protects DSS-induced acute colitis. Interestingly, in order to determine whether necroptosis contributes to colitis and CAT in MLKL−/−mice, phosphorylated MLKL was detected using Western blot analysis. They showed that phosphorylated MLKL was not found in AOM + DSS-induced CAT model (Figure 12). Altogether, implying that MLKL protects colitis and colitis-associated colon cancer through non-necroptotic function.

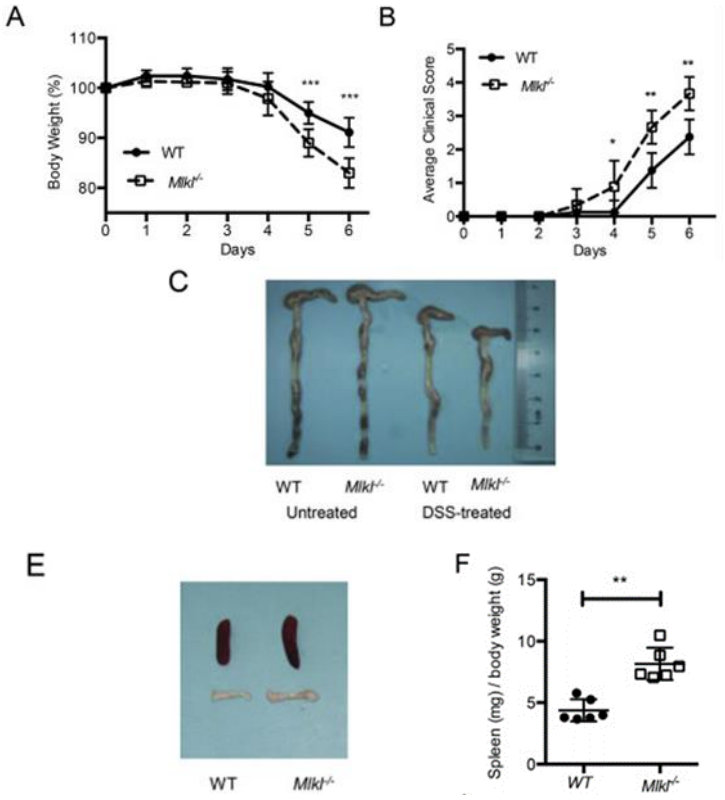

Figure 11 (A) Body weights (B) Average clinical score (C) colon length (E-F) Spleens and mesenteric lymph nodes (mLN) of WT and MLKL−/− mice were fed 3% DSS for 6 days

(45).

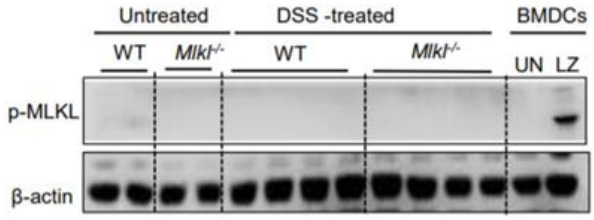

Figure 12 Phosphorylation of MLKL(p-MLKL) in AOM + DSS-induced CAT model (45)

2.5.4.2 NECROPTOSIS AND INFLAMMATION IN PROMOTING CANCER PROGRESSION

Necroptosis might promote cancer progression by activating inflammation. Since plasma membrane rupture in necroptosis pathway resulting in releasing damage-associated molecular patterns (DAMPs) that can trigger inflammation. The physiological process of inflammation is that it can recruit immune cells to eliminate cancer that

have been suggested in case of breast cancer as mentioned above. However, if inflammation cannot be resolved, it will turn to chronic inflammation that contributes to initiate tumor growth and metastasis (46). For example, Xinjian Liu, *et al*., 2016 used CRISPR-Cas9 to knockout RIPK1, RIPK3 and MLKL in MDA-MB231 cell line, a triple-negative breast cancer cell line, to study the role of key necroptotic proteins in cancer. They found that upon stimulation with necroptosis inducer (TSZ), the percentage of phosphorylated MLKL, a specific marker of necroptosis activation, in all 3 knockout cell lines were significantly reduced when compared with the parental cells. RIPK3 knockout cells were the most significantly reduced in cell growth when compared to RIPK1 and MLKL knockout cells (Figure 13).

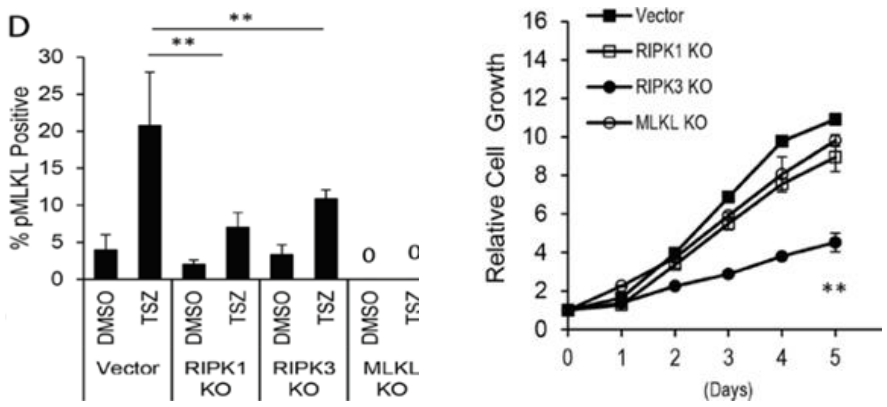

Figure 13 Left; % pMLKL positive in RIPK1, RIPK3 and MLKL knockout cells. Right; Cellular growth of RIPK1, RIPK3 and MLKL knockout cells (46)

Moreover, soft agar assay was used to examine the tumor growth abilities of the cells in vitro. They found that all 3 knockout cell lines were significantly reduced soft agar colony formation compared with the parental cells (Figure 14). In addition, cytokine profiling array showed that multiple cytokines were missing in necroptotic gene knockout especially IL-6, IL-8, and CCL5 which are the cytokines that involve in tumor growth (Figure 15). Therefore, overall results indicated that the necroptotic genes might regulate important cytokines which required for tumor growth.

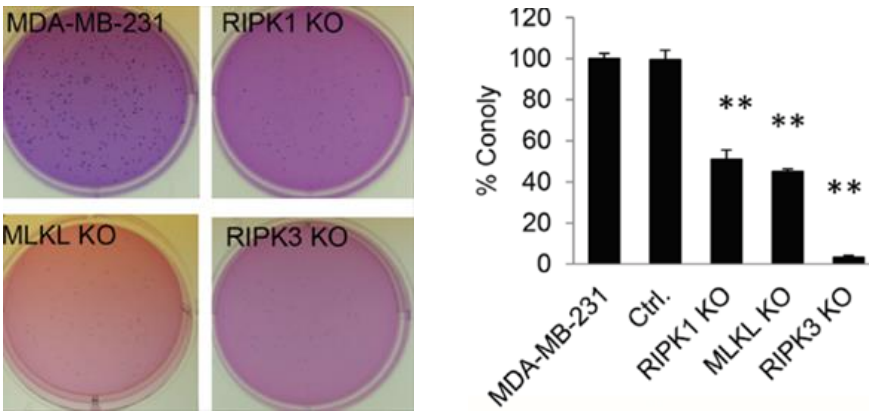

Figure 14 Measuring the tumor growth abilities of the cells in vitro using soft agar assay

(46)

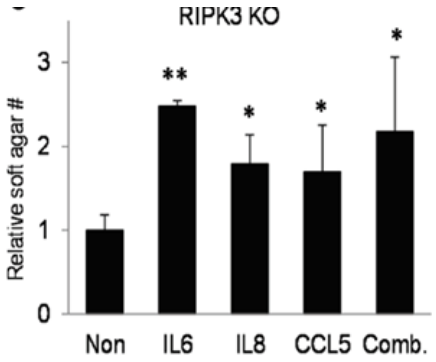

Figure 15 The result of cytokine profiling array

(46)

In addition, Yohei A, *et al*., 2020 reported that necroptosis releases CXCL5 to enhance migration and invasion of pancreatic cancer cells (Figure 16, 17) (47).

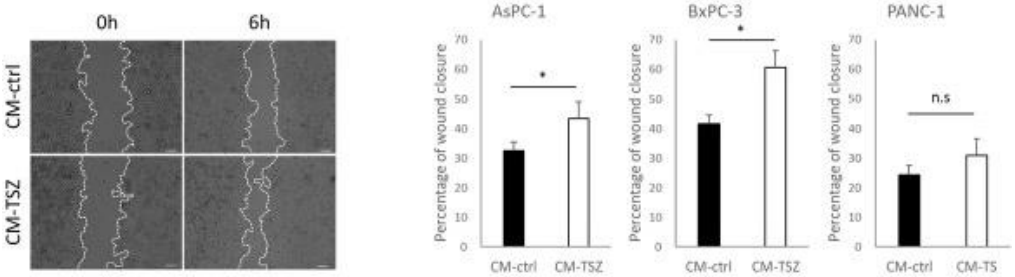

Figure 16 % Wound closure of pancreatic cells after treated with culture medium derived from necroptotic cells

(47)

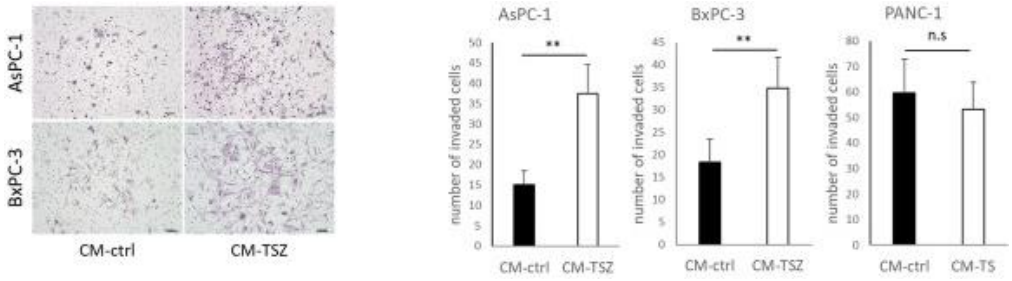

Figure 17 The number of invaded cells of pancreatic cells after treated with culture medium derived from necroptotic cells

(47)

2.5.4.3 NECROPTOSIS AND IMMUNOSUPPRESSION IN PROMOTING CANCER PROGRESSION

Necroptosis can promote cancer progression by inhibiting tumor immunity. Seifert L, *et al*.,2016 reported that RIPK3-mediated necroptosis promotes immunosuppressive tumor environment (TME) in pancreatic ductal adenocarcinoma (PDA) though enhancing tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) by releasing CXCL1 and SAP130 chemokine (Figure 18) (48, 49).

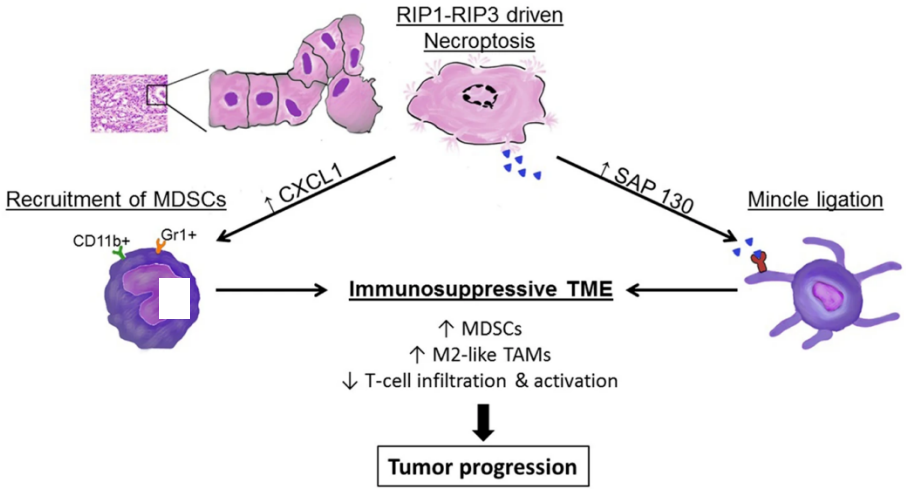

Figure 18 Schematic diagram showing RIPK3-driven necroptosis creates immunosuppressive TME in PDA

(48, 49)

However, those studies mentioned above are based on genetic deletion of key necroptotic proteins. Therefore, specific markers of necroptosis activation such as phosphorylated MLKL (pMLKL) should be further investigated to discriminate whether the effects on cancer are the results of necroptosis activation or its key molecules with non-necroptotic function.

2.6 NON-NECROPTOTIC FUNCTION OF MLKL

2.6.1 ROLE OF MLKL IN NERVE REGENERATION

In 2018, Ying *et al*. reported that MLKL plays role in promoting nerve regeneration after sciatic nerve injury through function beyond necroptosis, indicated that upregulation of MLKL might be a promising new therapeutic target for nerve injury and demyelinating diseases such as Amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease (CMT), and Multiple sclerosis (MS) (50).

2.6.2 ROLE OF MLKL IN REGULATION OF ENDOSOMAL TRAFFICKING AND GENERATION OF EXTRACELLULAR VESICLES

In 2017, Seongmin Y, *et al.,* reported that MLKL regulated endosomal transport through regulating the generation of intraluminal and extracellular vesicles (i.e. exosomes) through non-necroptotic function. Exosomes have been shown to serve as functional mediators in cellular communication which contribute to tumor progression, immunosuppression, and therapeutic resistance (Figure 19, 20) (51).

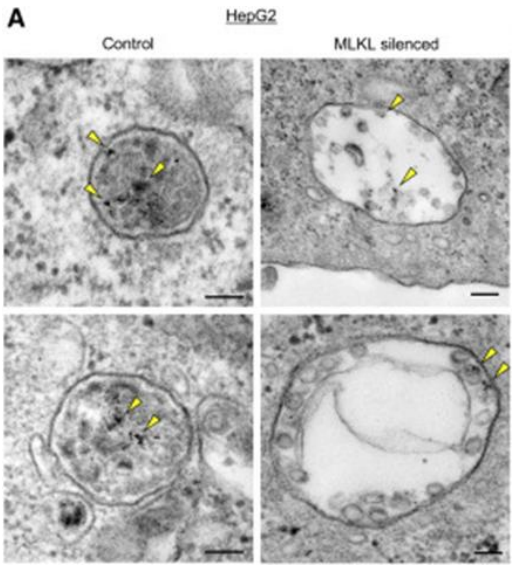

Figure 19 Transmission electron microscopy (TEM) of the multivesicular bodies in wild-type and MLKL knockdown HepG2 cells

(51)

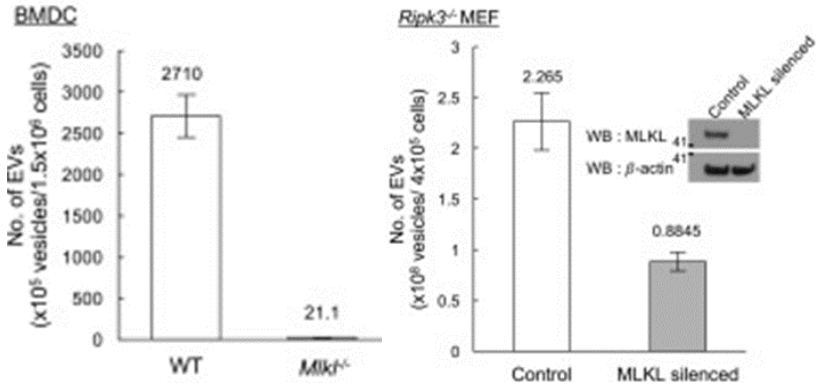

Figure 20 Effects of MLKL on extracellular vesicles generation

(51)

2.6.3 THE ENDOSOMAL TRAFFICKING ROLES OF MLKL ON TRAIL-INDUCED CELL DEATH

Recent data suggest that depletion of MLKL enhances TRAIL-induced cell death which is independent of necroptosis function. Mechanistically, they observed that depletion of MLKL reduces receptor-ligand endosomal trafficking leading to increased TRAIL cytotoxicity (8).

2.6.4 ROLE OF MLKL IN REGULATION OF ADHESION MOLECULE EXPRESSION IN ENDOTHELIAL CELLS

MLKL was suggested to modulate the expression of adhesion molecules (ICAM1, VCAM1, E-selectin) in endothelial cells through non-necroptotic function, subsequently lead to promote endothelial cell-leukocyte interaction during acute inflammation. (Figure 21) (52)

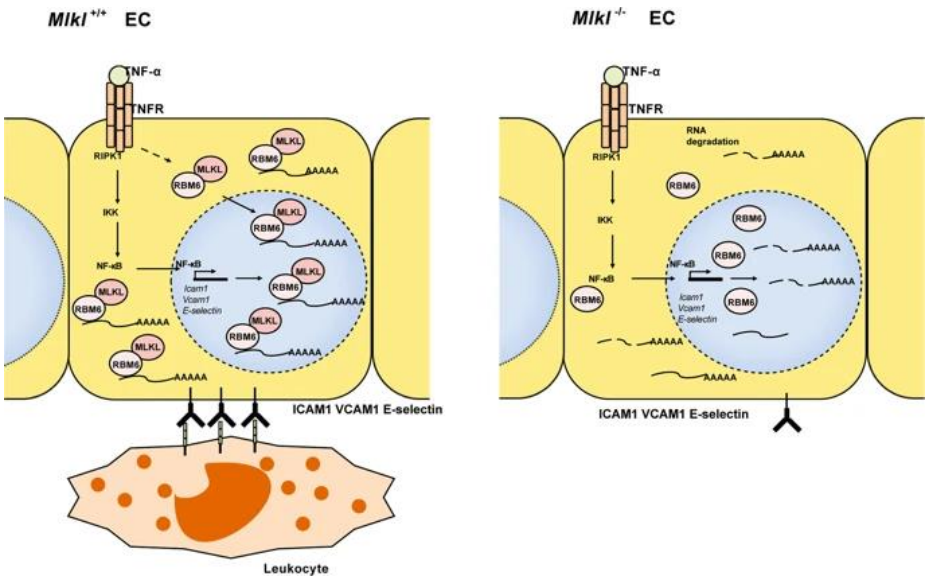

Figure 21 Conclusion model of MLKL in controlling the expression of adhesion molecules (ICAM1, VCAM1, E-selectin) in endothelial cells (52)

2.6.5 ROLE OF MLKL IN HEPATIC STELLATE CELLS

MLKL was highly expressed in hepatic stellate cells (HSCs) which contributes to hepatic fibrosis and that MLKL inhibition delays wound healing and vimentin expression in HSCs (9).

2.6.6 ROLE OF MLKL ON EMT IN RADIORESISTANT NASOPHARYNGEAL CARCINOMA CELLS

A recent study in nasopharyngeal carcinoma (NPC) cells has demonstrated that the depletion of MLKL expression inhibits invasion and significantly increases epithelial

markers (E-Cadherin) and decreases mesenchymal markers (Vimentin, N-Cadherin, Zeb1) of radioresistant NPC cells, independent of its role in necroptosis (10).

2.7 THE EXPRESSION OF RIPK3 & MLKL IN CANCER

As we mentioned earlier that the most studied and understood role of RIPK3 and MLKL are the key effector of necroptosis pathway, therefore they might play a positive role by eliminating tumor cells through necroptosis. Therefore, multiple solid cancer cell lines and primary tumor tissues are acquired ability to reduce RIPK3 and/or MLKL expression to evade necroptosis for their survival.

Table 1 The expression and prognosis of RIPK3 in cancer

Type of cancer	RIPK3 expression	Effect on Prognosis	References
Breast Cancer	Decreased	Poor prognosis	(44, 53)
Colorectal Cancer	Decreased	Shorter OS and DFS	(54)
Acute Myeloid Leukemia (AML)	Decreased	Poor prognosis	(55, 56)
Pancreatic ductal adenocarcinoma (PDA)	Increased	Promoted immunosuppressive TME and PDA progression	(48)
Non-small cell lung cancer (NSCLC)	Increased	Shorter OS	(57)
Low-grade glioma (LGG)	Increased	Shorter OS and DFS	(58)
Glioblastoma multiforme (GBM)	Increased	Shorter OS and DFS	(58)

Table 2 The expression and prognosis of MLKL in cancer

Type of cancer	MLKL expression	Effect on Prognosis	References
Pancreatic ductal adenocarcinoma (PDA)	Increased	Promoted immunosuppressive TME and PDA progression	(11)
Ovarian cancer	Decreased	Shorter OS	(12)
Cervical cancer	Decreased	Shorter OS	(13)
Gastric cancer	Decreased	Shorter OS	(14)
Colon Cancer	Decreased	Shorter OS	(15)
Esophageal squamous cell carcinoma (ESCC)	Increased	Shorter OS	(59)
Head and neck squamous cell carcinoma (HNSCC)	Increased	Shorter OS	(60)
Low-grade glioma (LGG)	Increased	Shorter OS and DFS	(58)
Glioblastoma multiforme (GBM)	Increased	Shorter OS and DFS	(58)

Many studies reported that there are 2 possible mechanisms that might be the cause of loss of RIPK3 expression in cancer which are epigenetic DNA modification and genetic mutation, however the mechanism depends on cancer types (61-63). While the underlying mechanisms regulate the loss of MLKL expression in cancer are still unknown and should be further investigation.

2.8 MLKL IN CANCER

Since MLKL is an executioner of necroptosis and downstream target of RIPK3 in necroptosis pathway, the level of MLKL expression might indicate the intensity of

necroptosis pathway in cancer. Previously studies have reported that MLKL serves as a good prognostic biomarker for patients with several cancers such as early-stage resected pancreatic cancer (11), ovarian cancer (12), cervical cancer (13), gastric cancer (14), and colon cancer (15). Low MLKL expression was significantly associated with both shorter disease-free survival (DFS) and overall survival (OS) in patients with these cancers. Therefore, these studies indicated that MLKL might serve as a good prognostic biomarker for patients with these cancers. One possible mechanism that low MLKL expression was associated with poor prognosis of these cancer patients is decreasing of necroptosis pathway, resulting in cancer cells will survive from elimination and finally patients will get a poor prognosis. However, the underlying mechanisms and the functional roles of MLKL associated with prognosis of patients in these cancers are still unknown and must be further investigated.

Nevertheless, Wei Sun, *et al.*, 2019 using Oncomine gene co-expression analysis demonstrated that FA2H, an enzyme that required for synthesis the building blocks of sphingolipids and glycosphingolipids in neural tissue and epidermis, was significantly associated with MLKL expression in many cancers (Figure 22) (63). Moreover, they found that low FA2H was associated with poor prognosis of gastric cancer (GC) patients (Figure 32). Similar results were observed in MLKL, suggesting that FA2H might be a downstream target of MLKL in GC. However, the mechanism of MLKL and FA2H associated with prognosis of GC patients should be further investigated.

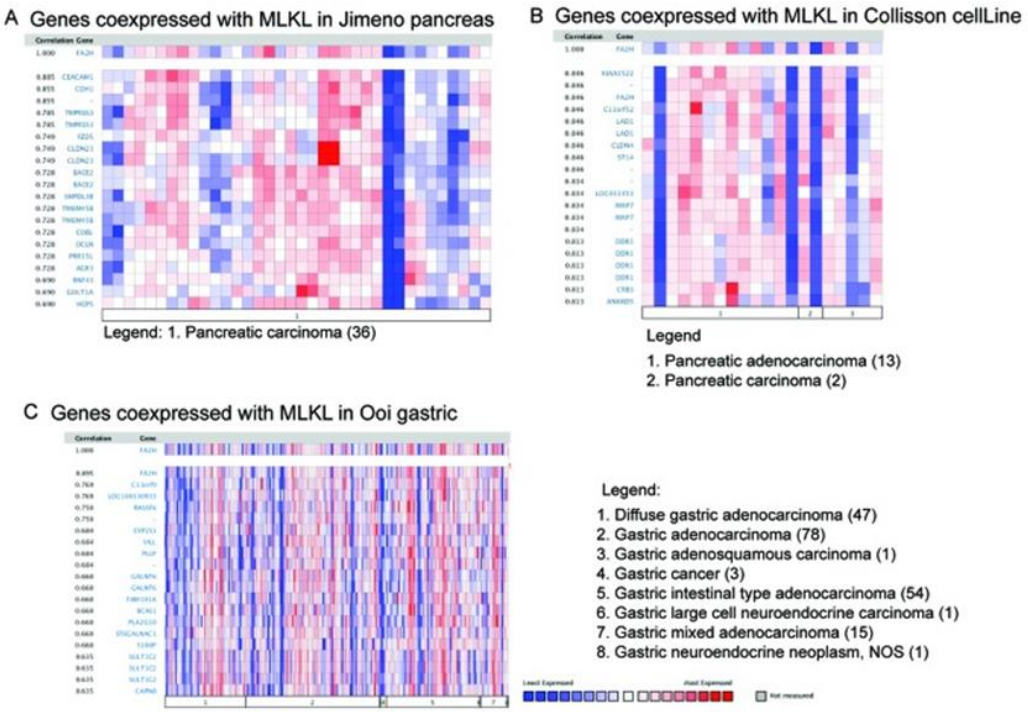

Figure 22 (A) Genes co-expressed with MLKL in Jimeno Pancreas database (B) in Collisson Cell Line database (C) in Ooi gastric database

(64)

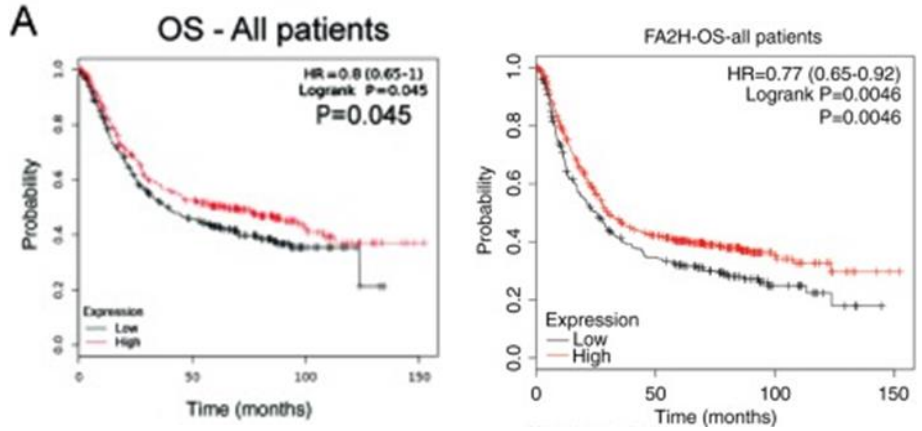

Figure 23 Prognostic values of MLKL Right; Prognostic values of FA2H in GC patients

(64)

2.9 MLKL IN CHOLANGIOCARCINOMA (CCA)

Nowadays, the expression pattern, survival and role of MLKL in CCA are still unknown. Therefore, in order to understand more about RIPK3 and MLKL in CCA, we

have analyzed RNA sequencing data from the public database at The Cancer Genome Atlas (TCGA) National Cancer Institute. They have performed RNA sequencing obtained from 36 cholangiocarcinoma samples. We found that RIPK3 and MLKL were highly expressed in human cholangiocarcinoma compared the normal bile duct tissues (Figure 24) (5). In addition, by collaboration with Tohoku University, Japan, our laboratory has performed immunohistochemistry analysis to look at RIPK3 and MLKL protein expression in 89 paraffin-embedded CCA tissue samples. We found that RIPK3 and MLKL expression in CCA tissues were significantly increased compared to normal bile duct (Figure 25, 26). However, the expression level of RIPK3 showed no difference between tumor and non-tumor area (Figure 25), while MLKL expression was significantly increased in CCA primary tissues compared to adjacent area (Figure 26). Therefore, these results suggested that MLKL might play a role in CCA.

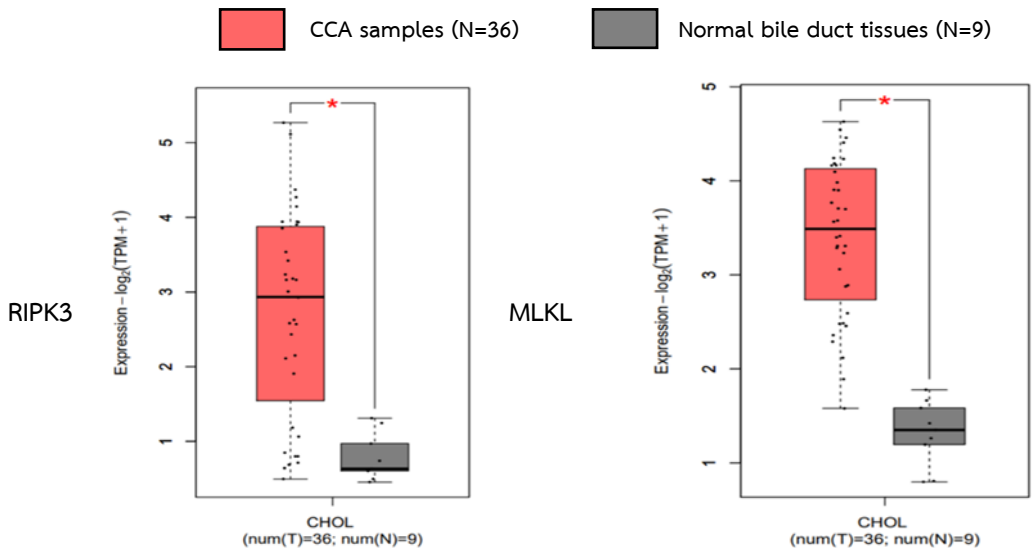

Figure 24 Expression of RIPK3 and MLKL in cholangiocarcinoma (TGCA database)

(5)

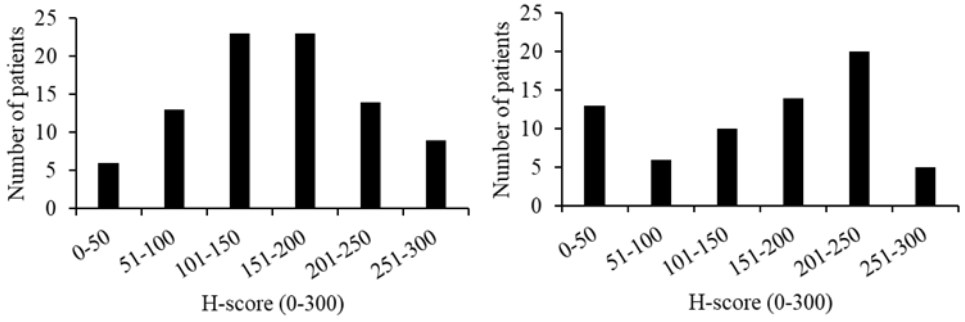

Figure 25 Differential expression of RIPK3 in cholangiocarcinoma primary tissues by Immunohistochemical analysis

(6)

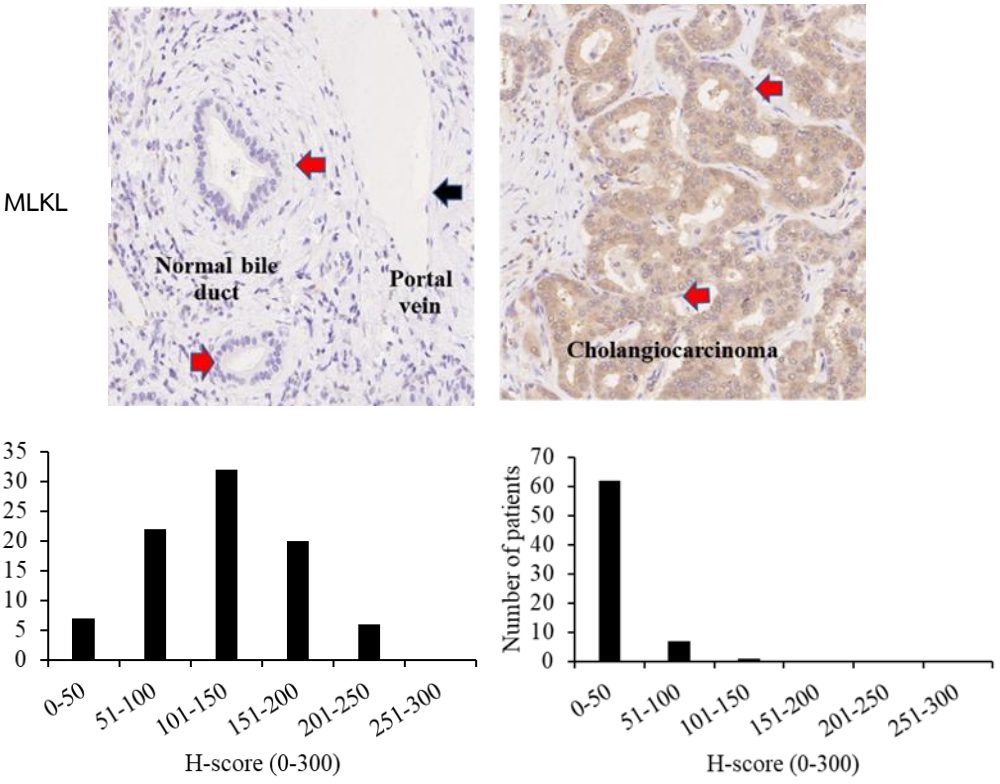

Figure 26 Upregulation of MLKL in cholangiocarcinoma by Immunohistochemical analysis

(6)

In addition, we found that high MLKL expression was significantly associated with both shorter in DFS and OS (Figure 27).

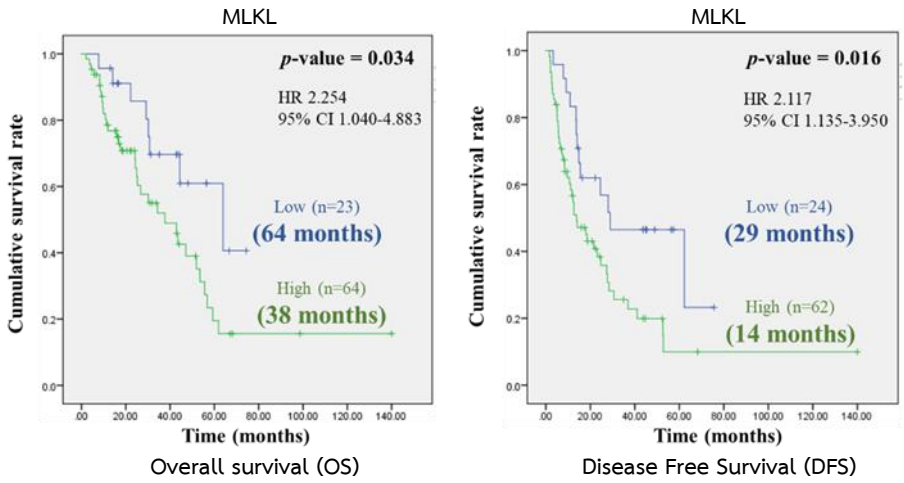

Figure 27 Survival analysis of MLKL in cholangicarcinoma (CCA) (6)

However, these results are contrast to the correlation between pMLKL which is a specific marker of necroptosis activation and survival rate of CCA patients that high pMLKL expression in CCA patients were associated with both longer in OS and DFS (Figure 28). Therefore, these results indicated that MLKL might have protumerigenic roles in CCA through function beyond necroptosis.

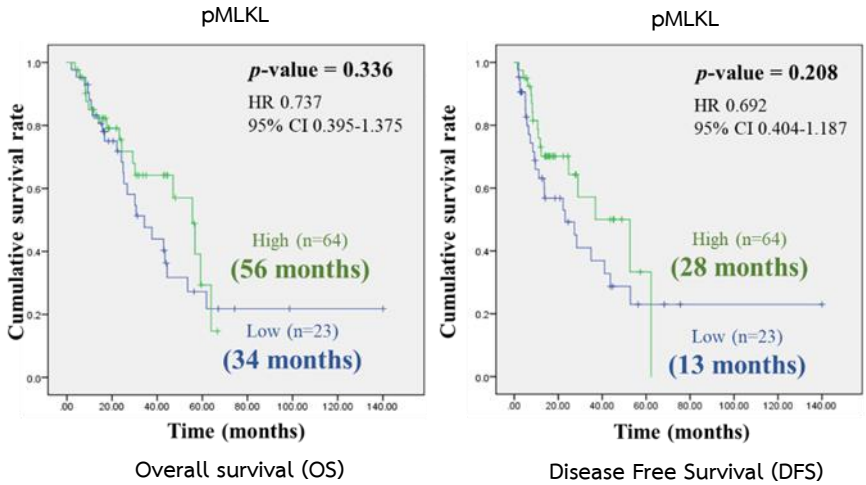

Figure 28 Survival analysis of pMLKL in cholangicarcinoma (CCA) (6)

Moreover, CCA is associated with chronic inflammation and immunosuppression which were known to influence the biological behavior of tumor and therapeutic outcomes. Therefore, in this study we used Pearson correlation to analyze the correlation between MLKL and $CD8^+$ T cells which is an anti-tumor leukocyte and tumor-associated macrophages (TAMs) which is a tumor promoting leukocyte. We found that MLKL expression was negatively correlated with $CD8^+$ T cells but positively correlated with TAMs which is contrast to the result of pMLKL (Figure 29). Therefore, these results suggested that high MLKL expression might promote pro-inflammatory responses in CCA tumor microenvironment.

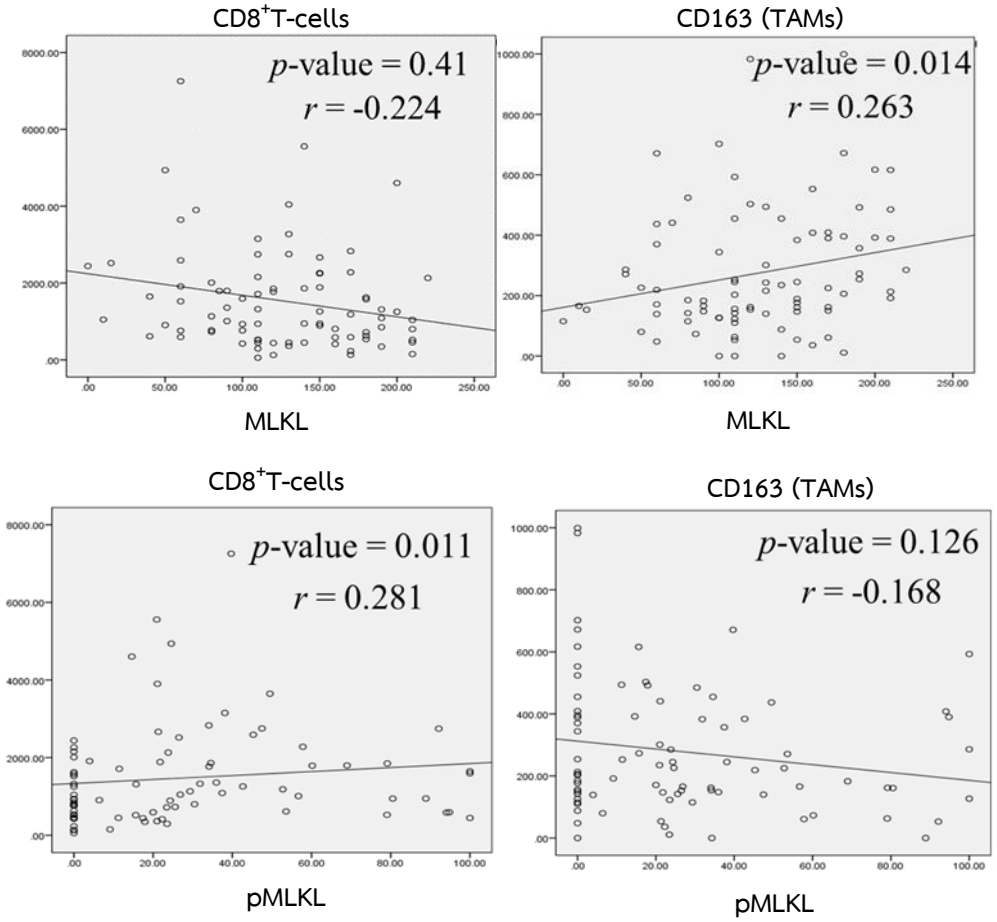

Figure 29 Correlation between MLKL& pMLKL and Tumor infiltrating leukocytes (TILs) using Pearson correlation method

(6)

Since MLKL was positively correlated with TAMs in which TAMs play a critical role in tumor progression in many aspects (52), this implied that the association between MLKL and TAMs might contribute to tumor progression. Hence, in this study we also analyzed the correlation between MLKL mRNA expression and proliferation, epithelial-mesenchymal transition (EMT), migration and invasion-related gene signatures using publicly available datasets and bioinformatic analysis. We found that MLKL was positively correlated with these hallmarks of cancer (Table 5,6).

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents

3.1.1.1 Reagents for cell culture

- Ham's Nutrient Mixture F12 medium (HyClone Laboratories, Logan, Utah, USA)
- Dulbecco's Modified Eagles Medium (DMEM) (HyClone Laboratories, Logan, Utah, USA)
- Penicillin/Streptomycin (HyClone Laboratories, Logan, Utah, USA)
- Fetal bovine serum (FBS) (Sigma, St Louis, Missouri, USA)
- Trypsin/0.25% EDTA (HyClone Laboratories, Logan, Utah, USA)
- Trypan Blue (Gibco-Invitrogen; Thermo Fisher Scientific, Inc., California, USA)

3.1.1.2 RNA interferences reagents

- pCMV-dr8.2-dvpr (Packaging plasmid) (Addgene#8455)
- pCMV-VSV-G (Envelope plasmid) (Addgene#8454)
- pLKO.1 puro (shNT plasmid) (Addgene#8453)
- Opti-MEM I (Gibco-Invitrogen; Thermo Fisher Scientific, Inc., California, USA)
- TurboFect transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., California, USA)
- Syringe-driven filters (0.45 μM) (JetBiofill, Guangzhou, China)
- Polybrene transfection reagent (Merck Millipore, Darmstadt, Germany)
- Puromycin (Merck Millipore, Darmstadt, Germany)

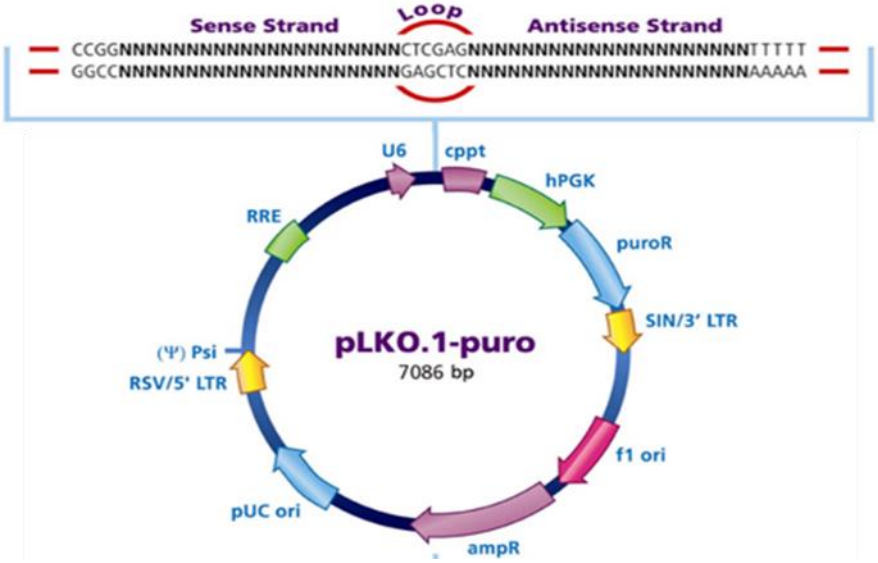

Figure 30 Lentiviral expression system: pLKO.1-puro plasmid (Sigma-Aldrich)

3.1.1.3 Reagents for MTT, colony formation and Propidium iodide (PI) hypotonic solution assay

- MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (AppliChem GmbH, Darmstadt, Germany)
- Dimethyl sulfoxide (DMSO), analytical grade (RCI labscan limited, Gillman SA, Australia)
- Methanol (RCI labscan limited, Gillman SA, Australia)
- Crystal violet (Sigma-Aldrich Pte. Ltd., Singapore)
- Propidium iodide (Invitrogen; Thermo Fisher Scientific, Lnc., California, USA)
- 10X PBS (HyClone Laboratories, Logan, Utah, USA)
- Sodium citrate (AppliChem GmbH, Darmstadt, Germany)
- Triton X-100 (AppliChem GmbH, Darmstadt, Germany)
- IsoFlow Sheath Fluid (1x10L) (Beckman Coulter, lnc, Atlanta, Georgia, USA)

3.1.1.4 Reagents for Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

- GENEzol Reagent (Geneaid, Taipei, Taiwan)

- Maxime™ RT PreMix Kit (Oligo dT15 Primer) (iNtRON Biotechnology lnc., Gyeonggi-do, Korea)

- iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, California, USA)

- Forward primer, 25nmole (U2BIO (THAILAND) CO., LTD., Bangkok, Thailand)

- Reverse primer, 25nmole (U2BIO (THAILAND) CO., LTD., Bangkok, Thailand)

- Agarose (Molecular Biology Grade) (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia)

3.1.1.5 Reagents for Western blot analysis

- Complete mini EDTA-free protease cocktail inhibitor (Roche, Mannheim, Germany)

- RIPA lysis buffer (Merck Millipore, Darmstadt, Germany)

- Bradford reagent (Bio-Rad, Hercules, California, USA)

- Standard bovine serum albumin (BSA) (AppliChem GmbH, Darmstadt, Germany)

- Tween-20 (AppliChem GmbH, Darmstadt, Germany)

- 30% Acrylamide/Bis Solution 29:1 (Bio-Rad, Hercules, California, USA)

- Ammonium Persulfate (APS) (Bio-Rad, Hercules, California, USA)

- β-mercaptoethanol (AppliChem GmbH, Darmstadt, Germany)

- Tetramethylethylenediamine (TEMED) (AppliChem GmbH, Darmstadt, Germany)

- Polyvinylidene Fluoride (PVDF) membrane 0.45 μM (Amersham, GE Healthcare, Buckinghamshire, United Kingdom)

- Precision Plus Protein™ Prestained Standards Ladder (Bio-Rad, Hercules, California, USA)

- Blotting-Grade Blocker (Bio-Rad, Hercules, California, USA)

- Clarity™ Western Enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, California, USA)

- UltraCruz® Autoradiography Film (Santa Cruz Biotechnology, lnc., Dallas,

Texas, USA)

- GBX Developer (KODAK)

- GBX Fixer (KODAK)

3.1.1.6 Antibodies for Western blot analysis

- Rabbit Anti-human MLKL monoclonal antibody (Abcam, Cambridge, United Kingdom), ab184718, Molecular weight 54 kDa, Dilution 1:5000

- Rabbit Anti-E-cadherin (24E10) monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA), cat no. #3195, Molecular weight 135 kDa, Dilution 1:5000

- Rabbit Anti-Vimentin (D21H3) monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA), cat no. #5741, Molecular weight 57 kDa, Dilution 1:1000

- Rabbit Anti-β-actin (13E5) monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA), cat no. #4970, Molecular weight 42 kDa, Dilution 1:5000

- Anti-rabbit IgG, Horse radish peroxidase (HRP)-linked secondary antibody (Cell signaling, Danvers, Massachusetts, USA), cat no. #7074

3.1.1.7 Reagents for Invasion assay

- Corning® Matrigel® Matrix (Corning Inc., New York, USA)

- Methanol (RCI labscan limited, Gillman SA, Australia)

- Crystal violet (Sigma, St Louis, Missouri, USA

3.1.2 Equipment and Instruments

- Steri-Cycle CO_2 Forma 371 Incubator, CO_2 incubator (Thermo Fisher Scientific, Inc., California, USA)

- Laminar flow biosafety cabinet class II (Haier Biomedical, Qingdao, China)

- Inverted microscope (Olympus Optical, Tokyo, Japan)

- Water bath (Memmert GmbH, Schwabach, Germany)

- Liquid Nitrogen Tank (Taylor-Wharton, Osaka, Japan)

- Hemacytometer (Hausser scientific, Horsham, Pennsylvania, USA)

- Flow cytometer, Navios (Beckman Coulter, Lnc, Atlanta, Georgia, USA)

- BioTek Synergy Mx reader (BioTek, inc., Winooski, Vermout, USA)

- Mini-Protein Tetra Vertical Electrophoresis Cell and Module (Bio-Rad, Hercules, California, USA)
- PowerPac™ HC high-current power supply (Bio-Rad, Hercules, California, USA)
- Block heater (Wealtec Corp., Way Sparks, Nevada, USA)
- Vortex Genie 2 (Scientific Industries, Bohemia, New York, USA)
- Refrigerated microcentrifuge (Hitachi, Tokyo, Japan)
- Tissue cell culture plates (96 wells/ 24 wells/ 12 wells/ 6 wells) and dishes (3.5 cm^2/ 6 cm^2/ 10 cm^2) (Corning Inc., New York, USA)
- Centrifuge tubes (15 mL/ 50 mL) and microcentrifuge tube (1.5 mL) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Cryovial tube 2.0 mL (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Round-bottom polystyrene tube 5 mL, 12x75 mm (BD Falcon Biosciences, San Jose, California, USA)
- Pipette tips (10 μL/ 200 μL/ 1000 μL) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Serological pipettes (5 mL/ 10 mL/ 25 mL) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., California, USA)

3.2 METHODS

3.2.1 Investigation of MLKL expression in panel CCA cell lines and CCA cell model selection

Research plan: To select two representative CCA cell lines, Western blot analysis was performed to examine the key necroptotic factors (RIPK1, RIPK3, and MLKL) expression in 7 different human CCA cell lines compared to a non-tumor human cholangiocyte cell line (MMNK-1).

3.2.1.1 CCA *in vitro* cell model

In order to examine the expression of RIPK1, RIPK3 and MLKL in CCA cell lines, 7 CCA cell lines as models of inflammation-associated cancer were used in this study. Six different available CCA cell lines have been established in Thailand including KKU100, KKU213 (KKU-213A), KKU214 (KKU-213B), KKUM055, HuCCA-1, and RMCCA-1 (65) and one CCA cell line which is HuCCT-1 has been established in Japan (66). MMNK-1 cell line, an immortalized cholangiocyte with SV-40 and hTERT transduction, was used as a comparative non-tumor cholangiocyte. All CCA cell lines were maintained in Ham's F12 medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin (Pen/Strep). All cell lines were maintained at 37 ºC in a 5% CO_2 humidified atmosphere.

3.2.1.2 MLKL expression by Western blot analysis

In order to examine the expression of RIPK1, RIPK3 and MLKL in CCA cell lines, cells were collected. Briefly, cells were collected in RIPA lysis buffer (50 mM Tris at pH7, 1% NP40, 150 mM NaCl, 0.5% Sodium Deoxycholate and protease inhibitor cocktail). The concentration of the lysates was measured by Bradford assay. Twenty-five μg of total protein was used to separate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 70 Volts 30 minutes for stacking gel and 100 Volts 60 minutes for separating gel. Next, separating proteins were transferred to PVDF membrane using 100 Volts 60 minutes and blocked with 1X TBST with 5% w/v nonfat dry milk. The membranes were incubated with Anti-MLKL antibody (1:5000, Abcam, ab184718) at 4°C overnight and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) at room temperature for 1 h. Finally, the membranes were developed by Enhanced Chemiluminescense system (ECL). The β-actin was used as internal control.

<u>Criteria to select CCA cells model</u>:

1. CCA cell lines which have high MLKL expression when compared to a non-tumor human cholangiocyte cell line (MMNK-1) were selected.

2. In order to investigate the role of MLKL on CCA invasion, CCA cell lines which have invasive phenotypes were selected.

3. CCA cell lines which express both RIPK3 and MLKL expression for future evaluation whether MLKL promotes CCA development and progression either through necroptotic or non-necroptotic roles were selected.

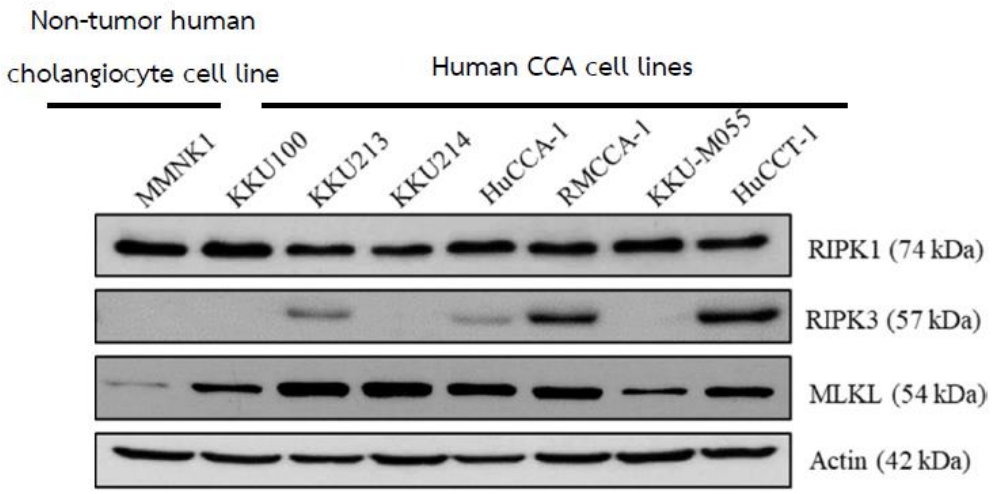

Figure 31 MLKL expression in seven differences human CCA cell lines compared to a non-tumor human cholangiocyte cell line (MMNK-1)

Similar to MLKL expression in primary CCA tissues, the expression of MLKL in all CCA cell lines were significantly increased compared to a non-tumor cholangiocyte, MMNK-1 (Figure 31). Therefore, KKU213 and HuCCT-1 which express high level of MLKL and RIPK3 and with invasive phenotypes were selected as CCA cell model to investigate the protumorigenic roles of MLKL in CCA *in vitro*.

3.2.2 Generation of MLKL knockdown CCA cell model using short hairpin RNA (shRNA)

Research plan: In order to investigate the protumorigenic roles of MLKL in CCA cell lines, inhibition of MLKL expression from CCA cells that express a functional MLKL was used to resolve these roles. In this study, loss of function genetic tools called RNA interference - short hairpin RNA (shRNA) was used to generate MLKL knockdown CCA cell model.

3.2.2.1 Selection the expression system

In order to introduce shRNA into MLKL- expressed CCA cell lines (KKU213 and HuCCT-1), Lentiviral expression system that was provided by Dr. Zhang's lab called pLKO.1-puro plasmid (commercially available from Sigma- Aldrich) (Figure 30) was selected and the expression of shRNA was controlled by U6 promoter.

The target sequences for shRNA-MLKL are as follow:

shRNA-MLKL#1: 5'-CCTCTGACAGTAACTTTGATA-3'

3'-GGAGACTGTCATTGAAACTAT-5'

shRNA-MLKL#2: 5'-GAAGCTTCACTGAGACGATTA-3'

3'-CTTCGAAGTGACTCTGCTAAT-5'

shNT (non-targeting vector):

5'-CAACAAGATGAAGAGCACCAA-3'

3'-GTTGTTCTACTTCTCGTGGTT-5'

3.2.2.2 Lentiviral production in HEK293T cells using liposome-mediated transfection

Since the expression system was lentiviral expression system, viral particles were produced in cells that are easy to transfect called HEK293T cells. First, HEK293T cells were cultured in serum free medium and transfected with 3 plasmids which are 1 μg of packaging plasmid (pCMV-dR8.2 dvpr), 0.125 μg of envelope plasmid (pCMV-VSV-G) and 1 μg of shRNA targeting MLKL or shNT using liposome-mediated transfection. After transfection, the supernatant-containing viral particles was collected and used to infect into MLKL expressing CCA cells (KKU213 and HuCCT-1).

3.2.2.3 Infection of Viral Particles into human CCA cell lines

First, MLKL expressing CCA cells (KKU213 and HuCCT-1) was cultured in completed culture medium for 24 h. Next, completed culture medium was changed

to serum free medium. Finally, the supernatant from 3.2.2.2 was added to infect MLKL expressing CCA cells including KKU213, and HuCCT-1 and the cells were then incubated in 5% CO_2 at 37 ºC for 24 h.

3.2.2.4 Selection of infected cells using puromycin antibiotics

Cells which were successfully infected with shRNA plasmids were selected by culture the cells in medium containing puromycin antibiotics (2 μg/ml), since shRNA plasmid contains puromycin resistant gene (Figure 30), therefore the cells which successfully infected with shRNA plasmids survived in medium containing puromycin antibiotics.

3.2.2.5 Validation of MLKL deletion using Western blot analysis

From the multiple sequence alignment results, shRNA-MLKL 1 and 2 sequences were aligned to the sequences of MLKL isoform 1 and 2, suggesting that isoform 1 and 2 of MLKL can be targeted by shRNA-MLKL 1 and 2. In addition, MLKL isoform 2 shared similarities to MLKL isoform 1, therefore RT-PCR could not be used to distinguish the isoforms of MLKL. Therefore, to test the effectiveness of the MLKL shRNA at the downregulation of MLKL expression, after 3 days of puromycin selection, Western blot analysis was performed to verify MLKL expression from pooled cells.

3.2.3 Investigation the protumorigenic roles of MLKL in CCA cell lines

Research plan: To investigate the functional roles of MLKL in MLKL knockdown CCA cell model, pro-tumorigenic properties including proliferation, migration, EMT, and invasion were investigated.

3.2.3.1 Bioinformatics analysis of MLKL mRNA expression and its correlation with protumorigenic gene signatures

Two transcriptome profiles from the Gene Expression Omnibus (GEO) database including GSE76297 and GSE107943 were collected in this study to analyze the association between MLKL mRNA expression and pro-tumorigenic gene signatures. GSE76297 and GSE107943 were downloaded from the Gene Expression Omnibus (GEO)

database (https://www.ncbi.nlm.nih.gov/geo/). The detailed information of CCA patients and the techniques used to identify their RNA expression profiles were shown in Table 3.

Table 3 Detailed information of transcriptome profiling of CCA patients obtained from the Gene Expression Omnibus (GEO) database

Datasets	CCA primary tissues	Normal bile duct tissues	Type of CCA	Techniques for mRNA expression profiling
GSE76297	91	91	iCCA	RNA microarray
GSE107943	30	27	iCCA	RNA sequencing

Gene signatures of each hallmarks of cancer were collected from Molecular Signatures Database (MSigDB) (www.gsea-msigdb.org/gsea/msigdb/) and the score of each gene signature of each CCA patient was calculated by bioinformatics tool "Gene Set Enrichment Analysis (GSEA)" (www.gsea-msigdb.org/gsea/index.jsp). Subsequently, Pearson correlation from SPSS software was used to analyze the correlation between MLKL mRNA and protumorigenic gene signatures.

3.2.3.2 Investigation the role of MLKL on CCA cells proliferation using MTT assay

3.2.3.2.1 Determination the optimal number of CCA cells for MTT assay

In order to investigate the role of MLKL on CCA proliferation by using MTT assay, optimal number of cells was determined. First, 1,000-5,000 CCA cells (KKU213 and HuCCT-1) were seeded into 96-well plates with 100 μL completed culture medium. After the specified incubation time (24, 48, 72, 96 h), 10 μL of MTT solution (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to each well and incubated at 5% CO_2 at 37 ºC for 2 h. After that, completed culture medium was discarded and 100 μL Dimethyl sulfoxide (DMSO) was add to each well to dissolve formazan. The absorbance value at 570 nm was measured using a microplate reader.

Criteria to select the optimal number of CCA cells in MTT assay

1. Absorbance value not higher than linearity.
2. Available space for cell growth which was determined under microscope.

3.2.3.2.2 Investigation the role of MLKL on CCA cells proliferation using MTT assay

In order to investigate the role of MLKL on CCA proliferation, the optimal number of MLKL knockdown CCA cells and shNT CCA cells were seeded into 96-well plates with 100 µL completed culture medium. After the specified incubation time (24, 48, 72, 96 h), 10 µL of MTT solution (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to each well and incubated at 5% CO_2 at 37 ºC for 2 h. After that, completed culture medium was discarded and 100 µL Dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan. The absorbance value at 570 nm was measured using a microplate reader and calculated % cell proliferation according to below formula:

$$\% \text{ Cell proliferation} = \frac{(Absorbance\ of\ shMLKL\ at\ 24{,}48{,}72\ or\ 96\ h)}{(Absorbance\ of\ shNT\ at\ 24{,}48{,}72\ or\ 96\ h)} * 100$$

3.2.3.3 Investigation the role of MLKL on CCA cells proliferation using colony formation assay

3.2.3.3.1 Determination the optimal number of CCA cells in colony formation assay

Before doing this experiment, optimal number of cells was determined. First, 100, 200, and 400 CCA cells (KKU213 and HuCCT-1) were seeded into 6-well plates with 1.5 mL completed culture medium. Next, cells were incubated at 5% CO_2 at 37 ºC. The number of cells which have formed sufficiently large colony (The colony is defined to consist of at least 50 cells) and colony was not overlapped to another colony were selected.

3.2.3.3.2 Investigation the role of MLKL on CCA cells proliferation using colony formation assay

Colony formation assay was used to confirm the role of MLKL on long-term proliferation. First, the optimal number of MLKL knockdown CCA cells and shNT CCA cells were seeded into 6-well plates. Next, cells were incubated at 5% CO_2 at 37 °C for 1-3 weeks until the cells can form the colony (The colony is defined to consist of at least 50 cells). Next, the culture medium was discarded and 1 mL of 1X PBS was added to wash the colony for 3 times. Then, the colony was fixed using 1 mL of 100% methanol for 15 minutes. Next, the colony was stained by using 1 mL of 1% crystal violet for 10 minutes. After that, the crystal violet was discarded, and stained colony was air-dry overnight. Finally, the colony was counted under microscope compared to shNT CCA cells.

3.2.3.4 Analysis of cell cycle phase using Direct DNA staining in Propidium iodide (PI) hypotonic solution

In order to analyze whether MLKL influences cell cycle phase, cell cycle of MLKL knockdown CCA cells was determined at 24, 48, 72 and 96 h compared with shNT CCA cells. First, MLKL knockdown CCA and shNT CCA cells were seeded in 3.5 cm^2 dish. The supernatant was collected in 1.5 mL microcentrifuge tube. Next, cells were washed with 1 mL of 1X PBS and 1X PBS was transferred to the same microcentrifuge tube. 200 μL of 0.25% EDTA-trypsin was added and cells was incubated at 37 °C for 5-10 minutes until the cells detach from the plate. Then, cells were collected into the same microcentrifuge tube and centrifuged at 2,000 rpm 5 minutes. Next, fix cells with 70% cold ethanol and put it on ice for 30 minutes and centrifuged at 2,000 rpm for 5 minutes. After that, the supernatant was discarded, and cells were washed with 1 mL of 1% BSA and centrifuged at 2,000 rpm 5 minutes. After that, the supernatant was discarded and 500 μL fluorochrome solution (PBS, 0.1% Triton X-100 and 50 μg/ml Propidium iodide (PI)) was added to lyse the cells and stained the DNA. Cells were incubated at room temperature in the dark for 1 h. Finally,

DNA content was analyzed using flow cytometry. The result of this assay was showed on histogram by Y-axis show the number of cells and X-axis show PI-intensity which represent DNA content (Figure 32). Cell cycle histogram of MLKL knockdown CCA cells was compared with histogram of shNT CCA cells.

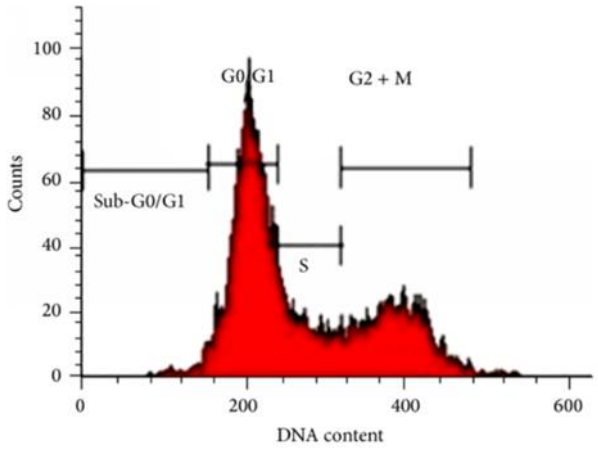

Figure 32 Cell cycle histogram

3.2.3.5 Investigation the role of MLKL on epithelial-mesenchymal transition (EMT) using qRT-PCR

Epithelial–mesenchymal transition (EMT) is a process that epithelial cells morphologically change into cells with a mesenchymal phenotype before cancer metastasis. (67) The characteristic of EMT process is decrease of epithelial cell markers (e.g. E-cadherin and Cytokeratin-19 (CK-19)) but increase mesenchymal markers (e.g. N-cadherin, Vimentin and Snail1). Hence, to determine whether MLKL regulates EMT process in CCA *in vitro*, qRT-PCR was used to examine the expression level of epithelial and mesenchymal cell markers in MLKL knockdown CCA cells compared to shNT CCA cells.

3.2.3.5.1 Screen epithelial-mesenchymal transition (EMT) markers in CCA cell lines

The expression level of 5 EMT markers which are epithelial markers including E-cadherin and Cytokeratin-19 (CK-19) and mesenchymal markers including

N-cadherin, Vimentin and Snail1 were analyzed by qRT-PCR in KKU213 and HuCCT-1. GAPDH was used as an internal control. Subsequently, one epithelial cell marker and one mesenchymal cell marker which have high gene expression in KKU213 and HuCCT-1 were selected to investigate the role of MLKL on EMT.

Table 4 Primer sequences for genes-associated EMT

Genes	Primer sequences
E-cadherin	Forward 5′-CGGCCTGAAGTGACTCGTAA-3’ Reverse 5′-AGAATCATAAGGCGGGGCTG-3’
Cytokeratin-19	Forward 5’-TGCCACCATTGAGAACTCCA -3’ Reverse 5’-TCAGGCCTTCGATCTGCATC-3’
N-cadherin	Forward 5’-GGTGTATGCCGTGAGAAGCT-3’ Reverse 5’-GTGGCCACTGTGCTTACTGA-3’
Vimentin	Forward 5′-CCTGTGAAGTGGATGCCCTT-3’ Reverse 5′-GTGACGAGCCATTTCCTCCT-3’
Snail1	Forward 5’-ATGCACATCCGAAGCCACA-3’ Reverse 5’-TTGTGGAGCAGGGACATTCG-3’
GAPDH	Forward 5’-ACATCGCTCAGACACCATGG-3’ Reverse 5’-ACCAGAGTTAAAAGCAGCCCT-3’

3.2.3.5.2 Investigation the role of MLKL on epithelial-mesenchymal transition (EMT) using qRT-PCR

In this experiment, qRT-PCR was used to determine the level of epithelial and mesenchymal mRNA in MLKL knockdown CCA cells compared to shNT CCA cells. First, total RNA was extracted from MLKL knockdown and shNT CCA cells using 1 mL of GENEzol reagent. 1 μg of RNA was converted into cDNA using Maxime™ RT PreMix Kit (Oligo dT15 Primer). Then, the mRNA quantitation of epithelial and mesenchymal cell markers in MLKL knockdown CCA cells were determined by qPCR assay with SYBR®

Green master mix. The relative epithelial and mesenchymal mRNA expression were calculated using the comparative Ct method and were represented as a ratio relative to the endogenous GAPDH mRNA level. All of the reactions were performed in triplicated, and the average Ct of target genes (epithelial and mesenchymal cell markers) and internal control (GAPDH) were calculated.

The ΔCt value was calculated by:

ΔCt = Average Ct of target gene – Average Ct of GAPDH

The ΔΔCT was calculated by:

ΔΔCt = ΔCt of MLKL knockdown CCA cells – ΔCt of shNT CCA cells

3.2.3.6 Investigation the role of MLKL on CCA migration using Wound healing assay

Wound healing assay is an easy and low-cost method to measure cell migration *in vitro*. The concept of wound healing assay is creation a gap called “scratch” on a confluent cell monolayer and the migration of cells will be captured at specified incubation time. Therefore, in order to determine whether MLKL regulates CCA migration *in vitro*, Wound healing assay was performed. Briefly, MLKL knockdown CCA and shNT CCA cells were seeded more than 90% confluency into 24 well-plates. Next, a yellow tip was used to create the scratch. After that, CCA migration was captured at 0, 6, and 12 h. Finally, % wound closure of MLKL knockdown CCA cells were calculated compared to shNT CCA cells according to below formula:

$$\% \text{ Wound closure} = \frac{(Length\ of\ scratch\ at\ 0\ h - Length\ after\ scratch)}{(Length\ of\ scratch\ at\ 0\ h)} * 100$$

3.2.3.7 Investigation the role of MLKL on CCA invasion using Transwell matrigel invasion assay

Transwell matrigel invasion assay is the assay that measures the capacity of cells to invade through extracellular matrix (ECM) *in vitro*. Therefore, in order to investigate the role of MLKL on CCA invasion, Transwell Matrigel invasion assay was

performed. In this experiment, there are 2 main components which are transwell insert and 24 well-plate that are separated by filtered membrane with 8 μm pore size. The pore size allows cancer cells to migrate into another side but in invasion assay cancer cells invade through ECM, therefore transwell membrane was coated with matrigel to mimic ECM. Next, 50,000 cells of MLKL knockdown CCA and shNT CCA cells were seeded onto the membrane and this transwell insert was put into 24 well-plate. Then, completed culture medium containing serum was added in 24 well-plate to act as chemoattractant to allow CCA cells invade into another side of insert. After that, the non-invaded cells were removed while the invaded cells were fixed using 500 μL of 100% methanol for 30 minutes. Finally, the invaded cells were stained by 300 μL of 0.1% crystal violet for 30 minutes and counted under microscope compared to shNT CCA cells.

3.2.4 Statistics and data analysis

All experiments were carried out in three independent experiments. The results were expressed as mean ± S.D. Student's t test was used to evaluate the statistical significance, and differences between mean values were considered significant when *p*-value is less than 0.05 (* represent $p < 0.05$, ** represent $p < 0.01$, *** represent $p < 0.001$).

CHAPTER IV

RESULTS

4.1 The expression of MLKL in CCA cell lines as representatives *in vitro* CCA cell models

In order to select CCA cell model and generate models of inflammation-associated cancer for *in vitro* functional studies. Both RIPK1 and RIPK3 that are key mediators in the same signaling pathway, necroptosis as MLKL were determined. Similar to MLKL expression in primary CCA tissues (5, 6), the expression of MLKL in all CCA cell lines were significantly increased compared to a non-tumor cholangiocyte, MMNK-1 in both mRNA and protein levels, while the expression of RIPK1 is similar in each CCA cell lines and MMNK-1, while only KKU213, HuCCA-1, RMCCA-1, and HuCCT-1 express RIPK3 (Figure 33, 34). Therefore, in this study, KKU213 and HuCCT-1 which are CCA cell lines expressing high level of MLKL and with invasive phenotypes were selected as CCA cell models to investigate the protumorigenic roles of MLKL in CCA *in vitro*.

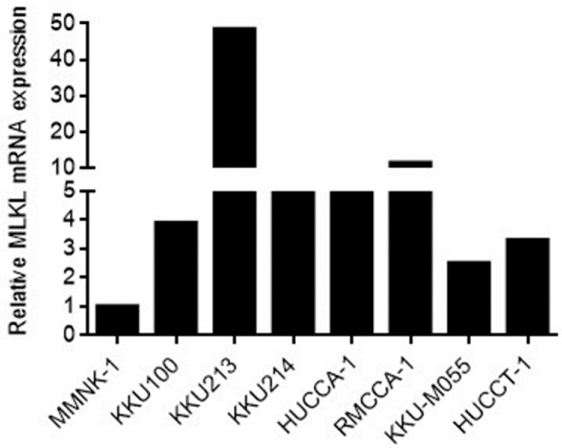

Figure 33 Expression of MLKL in 7 differences human CCA cell lines and a non-tumor human cholangiocyte cell line (MMNK-1) using qRT-PCR

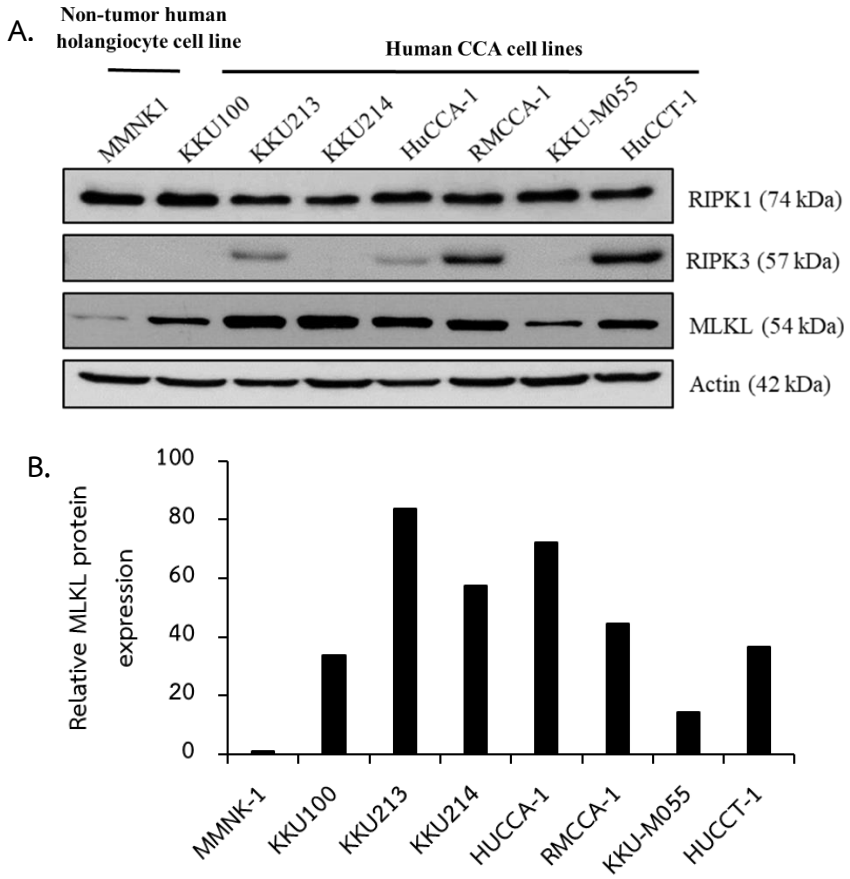

Figure 34 Expression of MLKL in 7 differences human CCA cell lines and a non-tumor human cholangiocyte cell line (MMNK-1) using Western blot analysis (5)

4.2 Generation of MLKL knock-down *in vitro* CCA cell model and cell model validation

In order to investigate the protumorigenic roles of MLKL in CCA cell lines, inhibition of MLKL expression from CCA cells that express a functional MLKL was used to resolve these roles. In this study, loss of function genetic tools called RNA interference - short hairpin RNA (shRNA) was used to generate MLKL knockdown CCA cell model. The procedure for a cell model generation was described in material and method (3.2.2). In this experiment, two different shRNA targeting MLKL were used to knockdown MLKL expression in KKU213 and HuCCT-1, and Western blot analysis was performed to validate MLKL inhibition from pool cells. As shown in figure 35A & 35B,

we found that MLKL expression was markedly decreased more than 90% in both KKU213 and HuCCT-1 after transfected with shRNA-MLKL#1 and shRNA-MLKL#2 compared with parental cells and shNT CCA cells. In addition, cell morphology of both KKU213 and HuCCT-1 MLKL knock-down cells are similar to shNT control, although both shRNA-MLKL#1 and shRNA-MLKL#2 KKU213 and HuCCT-1 cells grow slower than shNT control (Figure 35C). Additionally, we next functionally validated MLKL knockdown CCA cells by using a well-known function of MLKL which is the key component of necroptosis pathway. Therefore, shNT control and MLKL knockdown CCA cells were treated with TNF-α, Smac mimetic, and zVAD-fmk (TSZ) to induce necroptosis and treated with TNF-α and Smac mimetic (TS) to induce apoptosis for 24 h and detected cell death by AnnexinV and PI staining and flow cytometry. As expected, TSZ induced cell death in KKU213 shNT control, while cell death of KKU213 MLKL knockdown CCA cells were dramatically reduced (Figure 35D). In contrast to necroptosis, MLKL knockdown did not affect apoptosis which was induced by TS. These results suggest that the efficiency of MLKL knockdown is enough to functionally inhibit MLKL-induced necroptosis.

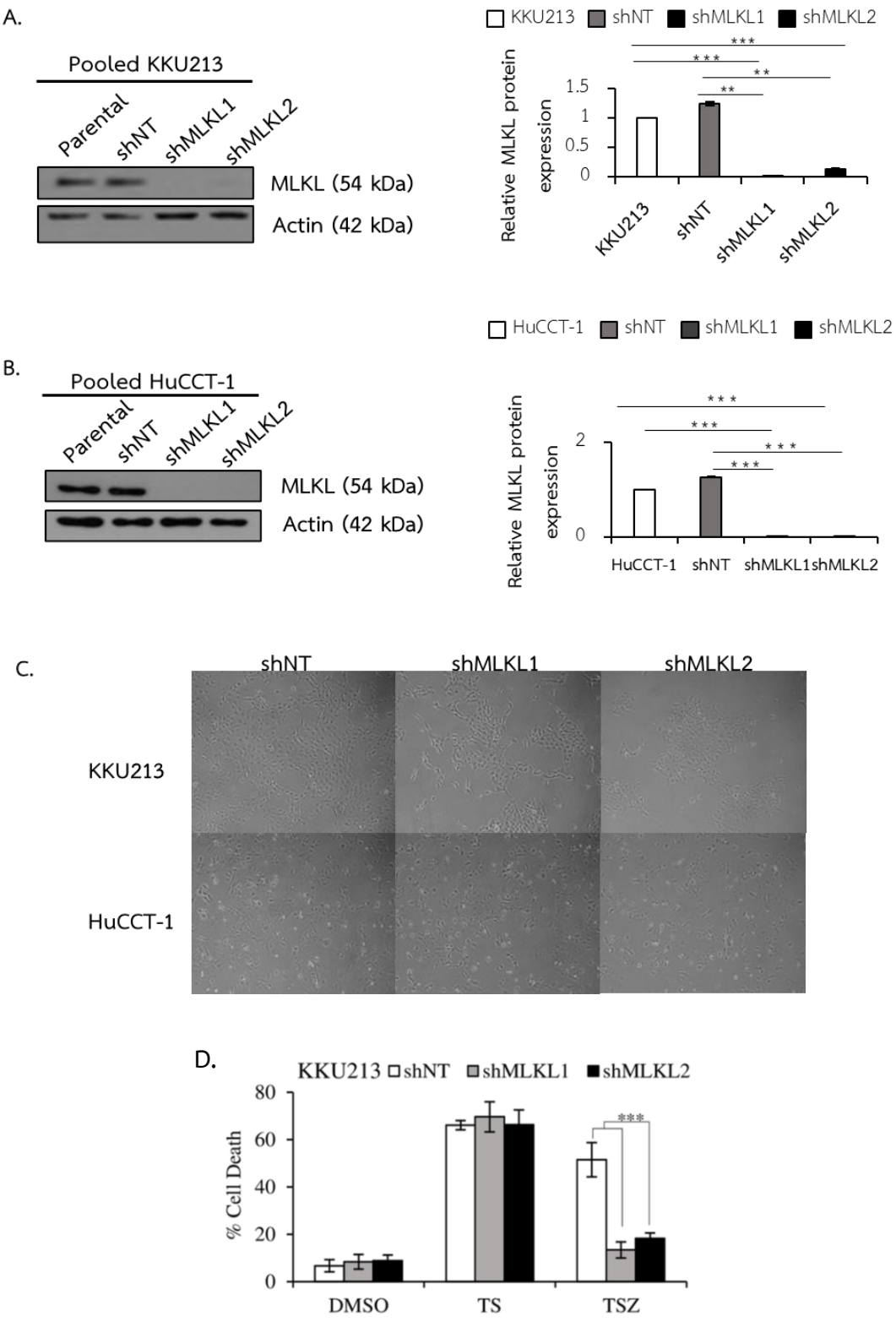

Figure 35 Generation of MLKL knockdown in vitro CCA cell model (KKU213 and HuCCT-1) and cell model validation

4.3 Investigation the pro-tumorigenic roles of MLKL in CCA cell lines

In order to validate our preliminary data obtained from clinical data and bioinformatics analysis from public database (mainly TCGA), we additionally analyzed two transcriptome profiles from the Gene Expression Omnibus (GEO) database including GSE76297 and GSE107943. In addition, the pro-tumorigenic roles of MLKL including proliferation, migration, EMT, and invasion in MLKL knockdown CCA cell models were investigated.

4.3.1 Bioinformatics analysis of MLKL and its correlation with protumorigenic gene signatures

We additionally collected two transcriptome profiles from the Gene Expression Omnibus (GEO) database including GSE76297 (N = 91) and GSE107943 (N = 30) to analyze the association between MLKL mRNA expression and gene signatures-associated with proliferation, epithelial-mesenchymal transition (EMT), migration and invasion. The detailed information of transcriptome profiling of CCA patients was shown in table 3. Our results in CCA demonstrated that MLKL expression was positively correlated with several genes-associated with hallmarks of cancer mentioned above (Table 5, 6). Therefore, these results further support the pro-tumorigenic roles of MLKL in CCA development and progression in which *in vitro* studies is needed.

Table 5 Correlation between MLKL and proliferation, epithelial-mesenchymal transition (EMT), migration and invasion gene signature from GSE76297 (N = 91)

Hallmarks of cancer	*r*	*p*-value
1. Proliferation-related signatures	0.584	1.19×10^{-9}
2. Epithelial-mesenchymal transition (EMT)-related signatures	0.264	0.011
3. Migration-related signatures	0.331	0.001
4. Invasion-related signatures	0.382	1.84×10^{-4}

Table 6 Correlation between MLKL and proliferation, epithelial-mesenchymal transition (EMT), migration and invasion gene signature from GSE107943 (N = 30)

Hallmarks of cancer	*r*	*p*-value
1. Proliferation-related signatures	0.536	0.002
2. Epithelial-mesenchymal transition (EMT)-related signatures	0.362	0.049
3. Migration-related signatures	0.405	0.027
4. Invasion-related signatures	0.474	0.008

4.3.2 Investigation the role of MLKL on CCA cell proliferation using MTT assay

Before investigation the role of MLKL on CCA proliferation by MTT assay, the optimal cell number for seeding KKU213 and HuCCT-1 in 96 well-plates were determined. We varied the cell number between 1,000-5,000 cells and measured the absorbance (OD) together with observed the morphology under microscope at 24-96 h. We found that 3,000 of KKU213 and 4,000 HuCCT-1 were the optimal number because the absorbance value was not higher than linearity, still have available space and minimal of cell death under microscope. (Figure 36A-C) Next, shRNA-MLKL#1 and shRNA-MLKL#2 in KKU213 and HuCCT-1 were seeded in 96 well plate and incubated for 24, 48, 72, 96 h. Interestingly, %cell survival in shRNA-MLKL#1 and shRNA-MLKL#2 in both KKU213 and HuCCT-1 were significantly decreased compared to shNT (Figure 36D). As observed under a microscope, a decrease in %cell survival is mainly due to a reduction in cell proliferation with a minimal effect on cell death.

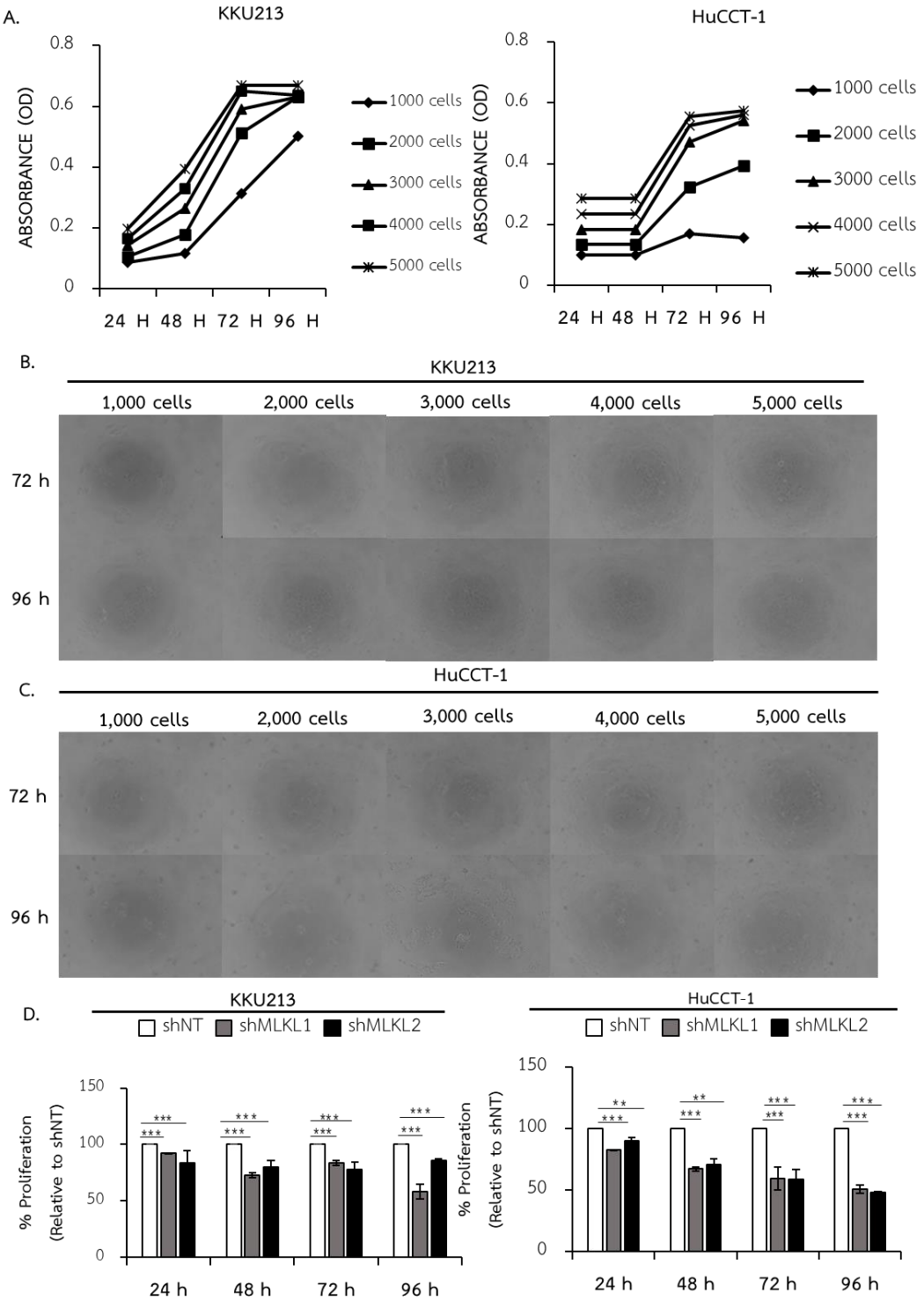

Figure 36 The effect of MLKL knockdown on CCA cell proliferation analyzed by MTT assay

4.3.3 Investigation the role of MLKL on CCA cell proliferation using colony formation assay

In order to investigate the role of MLKL on long-term CCA proliferation, colony formation assay was performed. Firstly, the number of cells was varied between 100, 200, and 400 cells for KKU213 and 1,000, 5,000, and 10,000 cells for HuCCT-1 and incubated for 14 days. The number of cells which have formed sufficiently large colony (The colony is defined to consist of at least 50 cells) and colony was not overlap to another colony were selected. 200 cells of KKU213 and 10,000 cells of HuCCT-1 were fit in these criteria. (Figure 37A) Then, 200 cells and 10,000 cells of MLKL-knockdown KKU213 and HuCCT-1 were seeded in 6-well plate and incubated for 14 days. As shown in figure 37B-C, consistent with MTT assay, the number of colonies in shRNA-MLKL#1 and shRNA-MLKL#2 in both KKU213 and HuCCT-1 were significantly decreased compared to shNT. These results indicate that MLKL might promote cell proliferation in CCA.

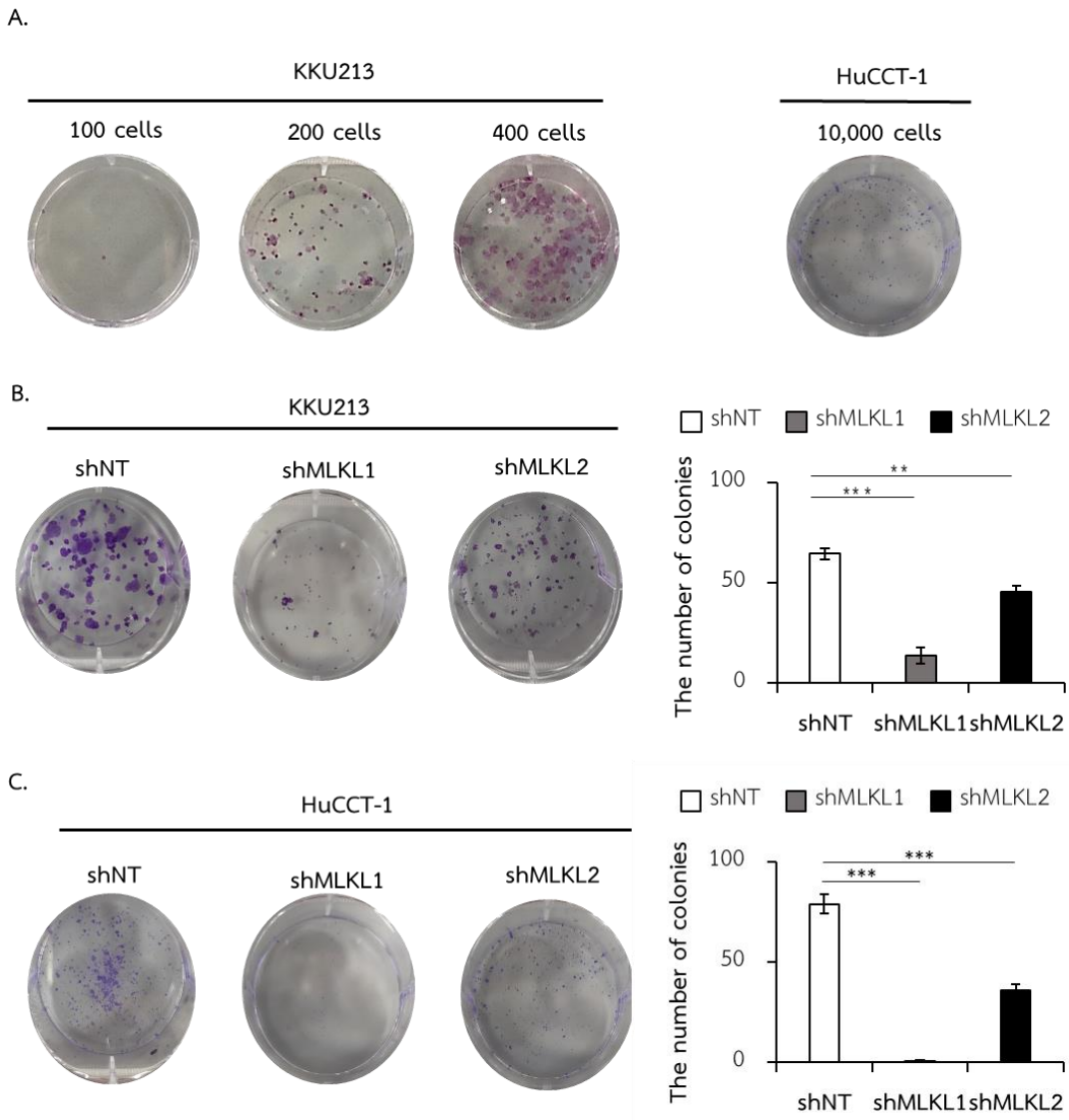

Figure 37 The effect of MLKL knockdown on long-term CCA cell proliferation analyzed by colony formation assay

4.3.4 Investigation the role of MLKL on cell cycle using flow cytometry

Since knockdown of MLKL inhibits cell proliferation as determined by both MTT and colony formation assay, to further investigate whether MLKL knockdown affects cell cycle phase and might contribute to cell proliferation inhibition, the analysis of cell cycle was determined by propidium iodide (PI) staining and analyzed by flow cytometry. As shown in figure 38, the percentage of cells in S phase was significantly increased in MLKL knockdown KKU213 cells at 48, 72, and 96 h compared to shNT.

This result suggests that MLKL might regulate cell cycle at S phase. The mechanisms underlying MLKL regulating cell cycle progression are needed for further studies, such as the expression levels of major cell cycle-promoting factors will be analyzed.

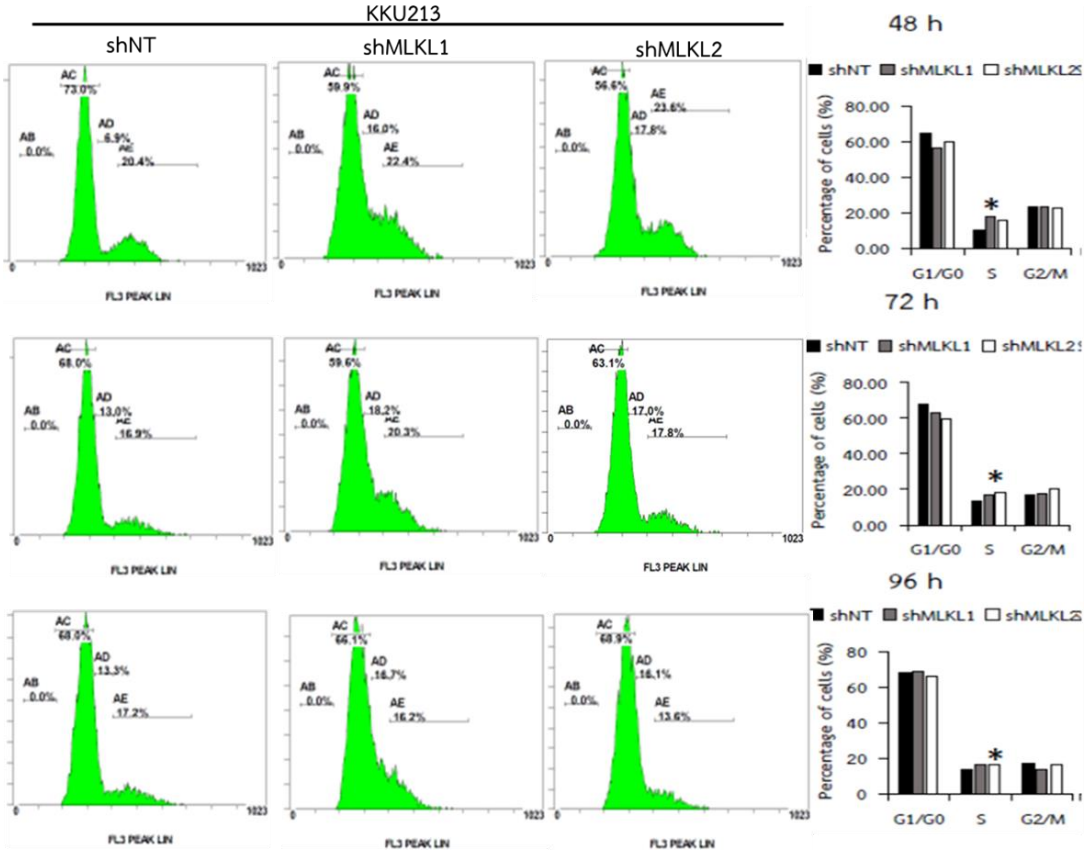

Figure 38 The effect of MLKL knockdown on cell cycle distribution analyzed by flow cytometry

4.3.5 Investigation the role of MLKL on CCA migration using Wound healing assay

We next investigated the effect of knockdown MLKL on CCA migration by using wound healing assay. After creation the artificial gap called “scratch” on MLKL knockdown KKU213 and HuCCT-1, the migration of CCA cells were captured at 0, 6, and 12 h, respectively and % wound closure of MLKL knockdown KKU213 and HuCCT-1 were calculated compared with shNT CCA cells. As shown in figure 39, on left the images were captured at the same frame. Quantitation of the wound healing experiments was carried out in which the average rate of wound closure was

calculated. Of great interest, % wound closure after 6 and 12 h was significantly lower in MLKL knockdown cells compared to shNT control. These results indicate that MLKL might involve in would healing process.

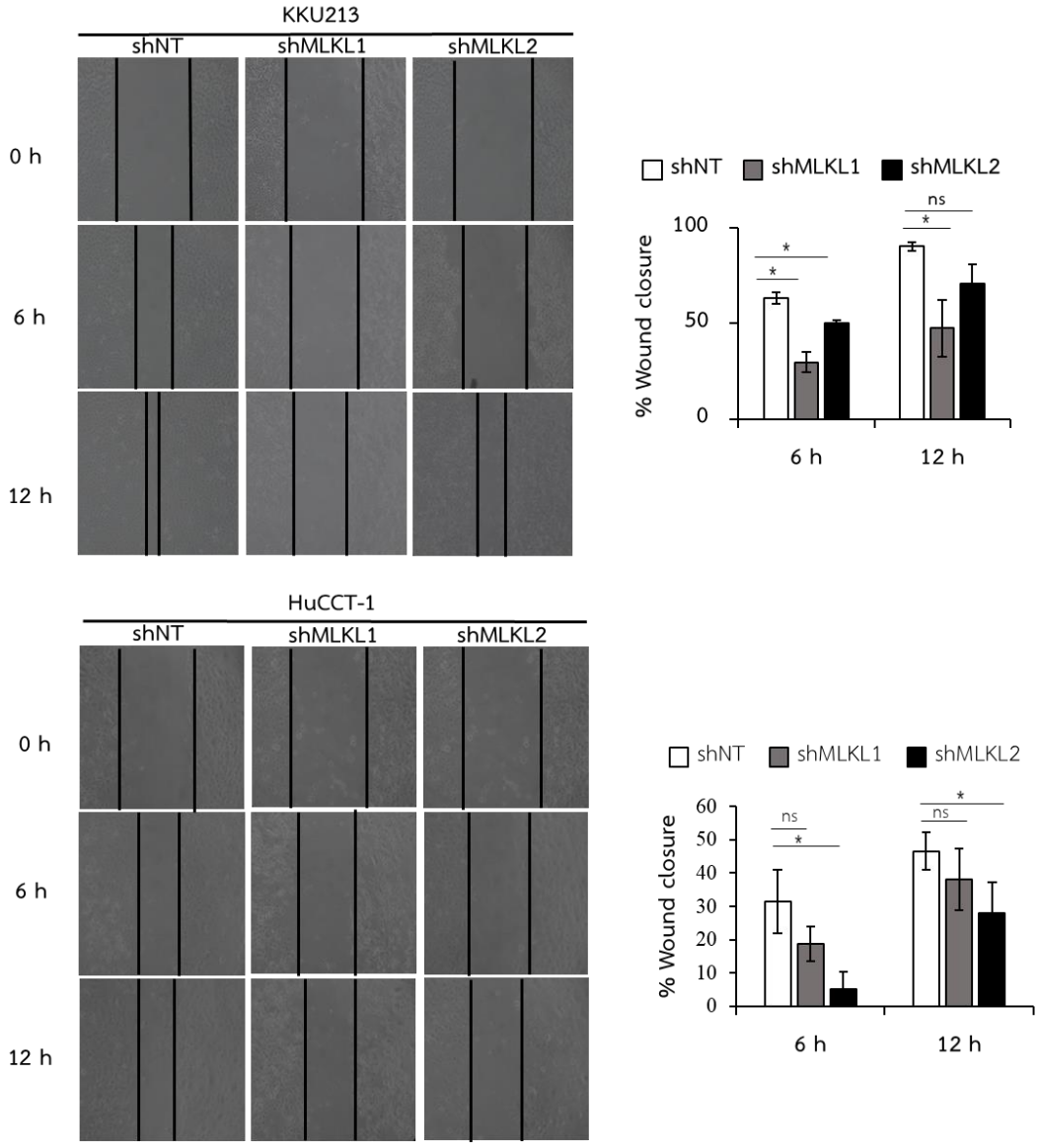

Figure 39 The effect of MLKL knockdown on cell migration analyzed by wound healing in KKU213 and HuCCT-1

4.3.6 Investigation the role of MLKL on CCA invasion using Transwell matrigel invasion assay

In order to determine the effect of knockdown MLKL on CCA invasion, Transwell matrigel invasion assay was performed and the number of invaded cells in MLKL knockdown CCA cells were counted at 6, and 12 h compared with shNT CCA cells. As shown in figure 40, the number of invaded cells was significantly lower in MLKL knockdown cells than in shNT after 12 h. This result indicates that MLKL might promote cell invasion in CCA cells.

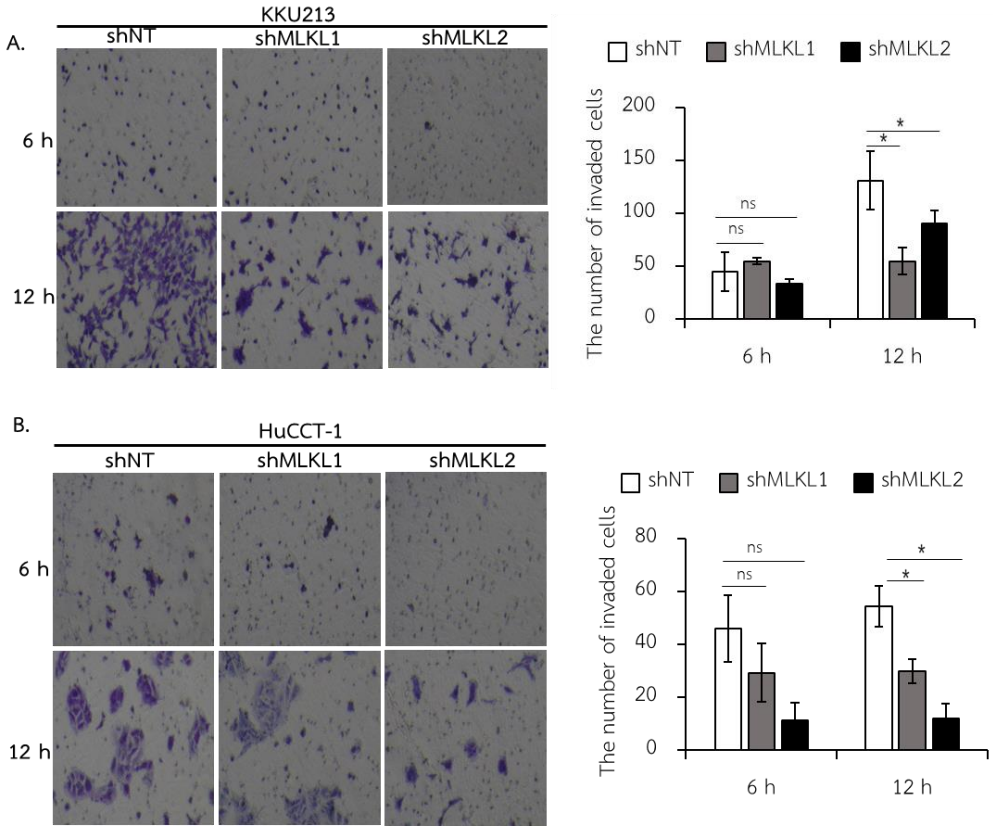

Figure 40 The effect of MLKL knockdown on invasion analyzed by Transwell matrigel assay in KKU213 and HuCCT-1

4.3.7 Investigation the role of MLKL on epithelial-mesenchymal transition (EMT) using qRT-PCR

To examine the expression of EMT-associated genes including epithelial (CK-19, E-cadherin) and mesenchymal (Snail1, vimentin, N-cadherin) were analyzed in KKU213

and HuCCT-1, gene expression was determined by qRT-PCR analysis. After that expression values were obtained by the delta Ct method, where geometric mean of GAPDH was utilized as the control gene expression values. Average expression values (2^ (-delta Ct)) x100 were plotted on the y-axis. As shown in figure 41, the strongest expression of CK-19, an epithelial marker and vimentin, a mesenchymal marker is observed in KKU213. Similar expression results are observed in HuCCT-1 (Figure 41). Since EMT properties as preliminary analyzed by gene expression analysis were not much changed in a resting condition compared to when stimulation with pro-inflammatory cytokines, therefore the roles of MLKL on EMT was mainly studied under TNF-α stimulation. To further examine whether stimulation with TNF-α could enhance EMT in KKU213 and HuCCT-1. KKU213 and HuCCT-1 were stimulated with 10 ng/ml TNF-α for 24, 48, and 72 h. As shown in figure 42, although the expression of E-cadherin was not significantly changed upon TNF-α stimulation at mRNA level, vimentin was significantly upregulated upon TNF-α stimulation in KKU213. In contrast to KKU213, TNF-α stimulation doesn't not induce a significant change in EMT markers in HuCCT-1 (Figure 43). Next, the expression of vimentin and E-cadherin was determined in MLKL knockdown KKU213 cells upon stimulation with TNF-α. As shown in figure 44, the expression of E-cadherin is not significantly changed. Interestingly, the expression of vimentin in KKU213 MLKL knockdown cells is significantly decreased compared to shNT. All together, these results indicate that MLKL might promote EMT in CCA cells, in which the underlying molecular mechanism needs further studies.

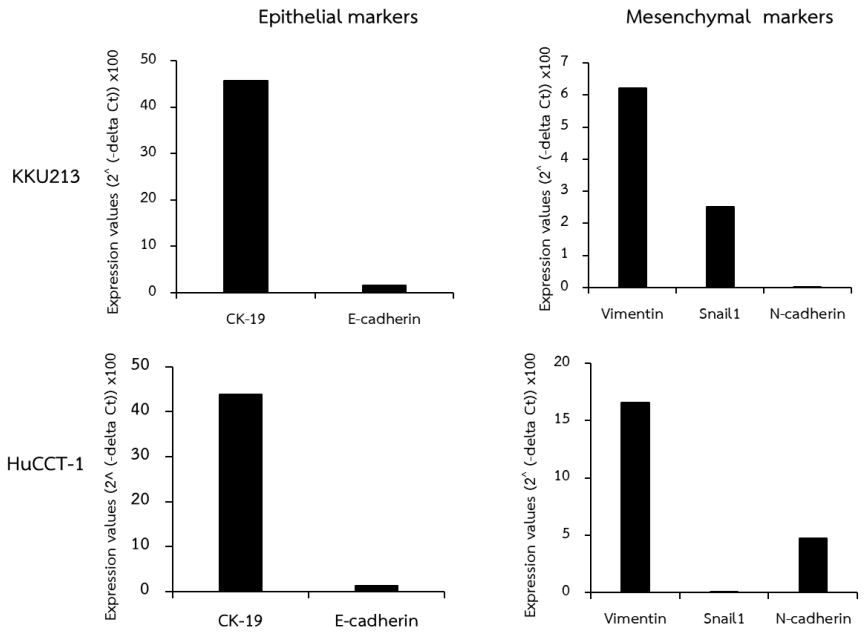

Figure 41 Relative gene expression of epithelial and mesenchymal markers in KKU213 and HuCCT-1

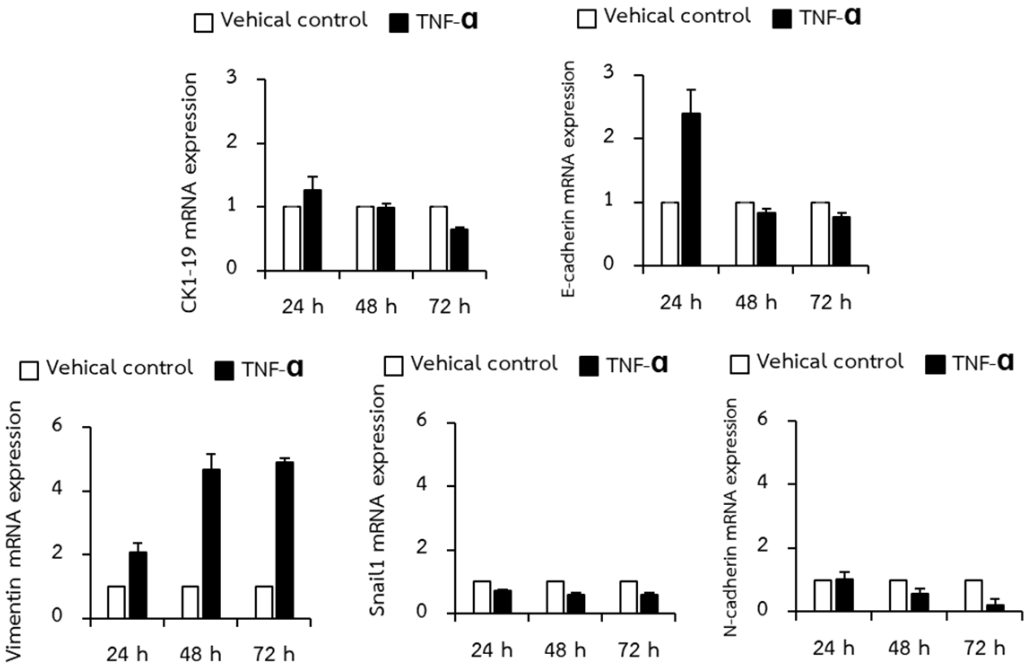

Figure 42 Relative gene expression of epithelial and mesenchymal markers upon stimulation with TNF-α in KKU213

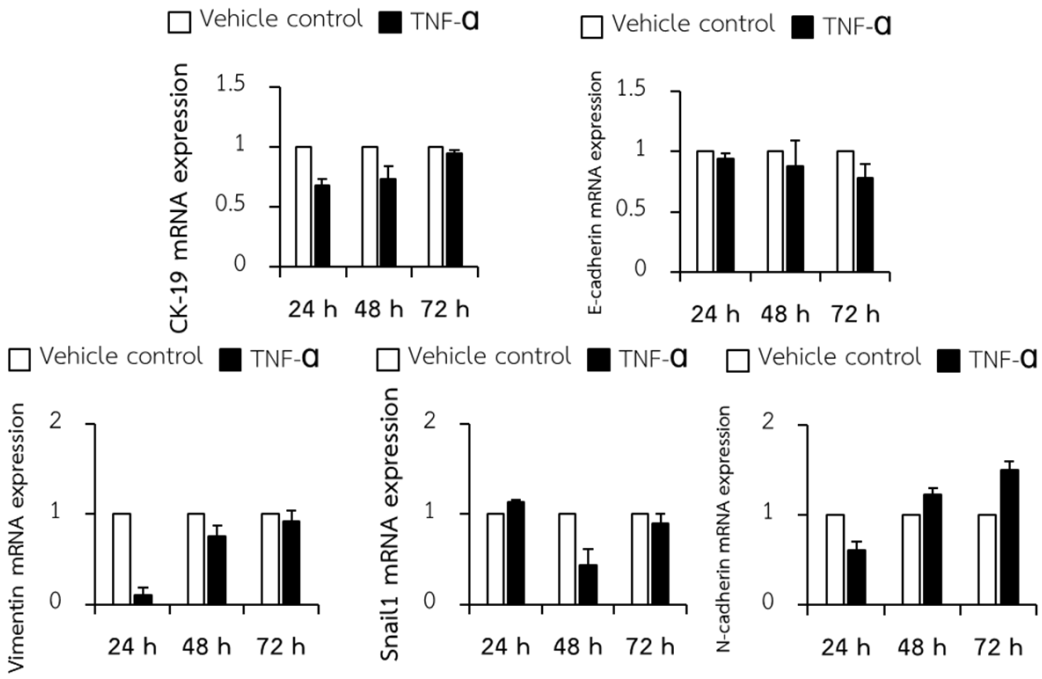

Figure 43 Relative gene expression of epithelial and mesenchymal markers upon stimulation with TNF-α in HuCCT-1

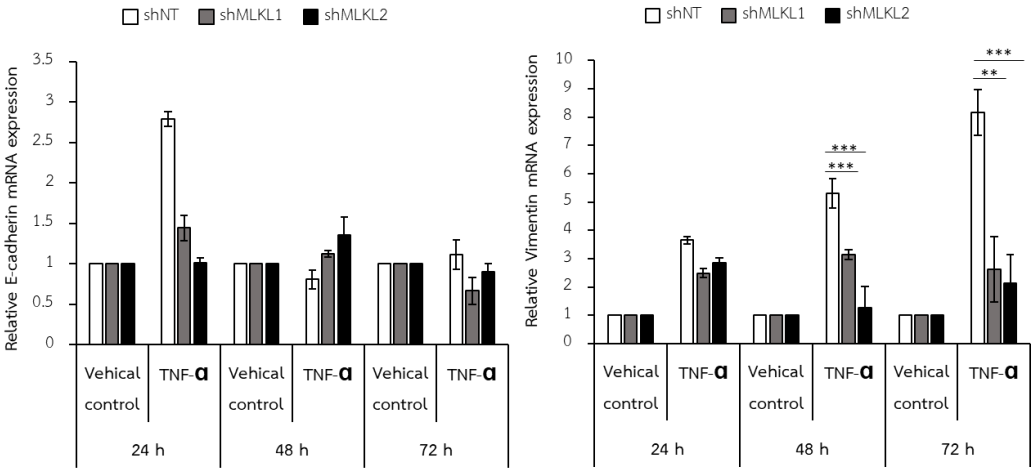

Figure 44 The effect of MLKL knockdown on epithelial and mesenchymal markers expression upon stimulation with TNF-α in KKU213

CHAPTER V

DISCUSSION AND CONCLUSION

In this study, we are the first study to investigate the pro-tumorigenic properties of MLKL including proliferation, migration, invasion, and EMT in CCA cells. Our results demonstrated that MLKL was significantly higher expressed in human CCA cell lines than a non-tumor cholangiocyte (MMNK-1) in both mRNA and protein levels. Interestingly, depletion of MLKL expression by short-hairpin RNA (shRNA) markedly reduced proliferation, migration, invasion, and EMT in CCA cells, which further support our finding in bioinformatics analysis, that MLKL mRNA expression was positively correlated with hallmarks of cancer gene signatures mentioned above in GSE76297 and GSE107943 cohorts. Taken together, our finding suggests that MLKL might have protumorigenic roles in CCA. Therefore, MLKL might be the new promising therapeutic target for CCA treatment.

Necroptosis, a regulated form of necrosis and an inflammatory form of cell death has been suggested to implicate in cancer through the release of intracellular contents including damage-associated molecular patterns (DAMPs) that can elicit marked inflammatory responses and adaptive immunity (29). Receptor-interacting protein kinase 1 (RIPK1), receptor-interacting protein kinase 3 (RIPK3), and mixed lineage kinase domain-like protein (MLKL) are three key components of necroptosis pathway. Nowadays, necroptosis and key necroptotic proteins have been suggested as a double-edged sword in cancer (68). First, as a role of cell death, necroptosis provides the new concept to overcome apoptosis resistance in cancer cells (68). Therefore, in order to evade necroptosis cell death, it is not surprised that downregulation of key necroptotic factors has been reported in several types of cancer such as colorectal cancer, breast cancer, acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL) (61). Additionally, their low expression was associated with shorter OS in these types of cancer, suggesting that necroptosis might play the anti-tumorigenic role in cancer. On the other hand, studies on esophageal squamous cell carcinoma (ESCC), head and

neck squamous cell carcinoma (HNSCC), pancreatic ductal adenocarcinoma (PDA), low-grade glioma (LGG), glioblastoma multiforme (GBM), non-small cell lung cancer (NSCLC) reported the opposite finding that upregulation of necroptosis-associated factors were related to unfavorable prognosis in these cancers (48, 57, 59, 60). It implies that the correlation between necroptosis and prognosis of patients may rely on the type of cancer, stage of cancer, and cellular context in tumor microenvironment (TME). Nowadays, RIPK1 and RIPK3 in cancer have been studied extensively, but little is known about the exact role of MLKL in cancer including CCA.

CCA has high mortality rate due to difficulty of diagnosis in early stage and lack of effective therapy. Therefore, a better understanding of molecular pathogenesis of CCA will lead to the development of a novel therapeutic target and strategies. Our previous study found that MLKL expression was upregulated in both mRNA and protein levels, which mRNA expression in CCA primary tissues were obtained from TCGA database and their protein levels were obtained from paraffin embedded CCA samples (5, 6). Similar result was observed in our study that MLKL was increased in both mRNA and protein level in panel human CCA cell lines compared to a non-tumor cholangiocyte (MMNK-1) (5). These results indicate that upregulation of MLKL might play roles in CCA. However, the regulatory mechanism controlling MLKL expression and its functional role in CCA are required for further study.

Since, both mRNA and protein expression of MLKL are higher in both CCA primary tissues and cell lines, one possible mechanism controlling MLKL expression might be regulated by epigenetic modifications, a major transcriptional control of gene expression. Moreover, our preliminary study suggested the interplay between MLKL and pro-inflammatory responses in CCA tumor microenvironment. We found that MLKL expression is associated with the inflammatory/immune cells infiltration in tumor microenvironment (TME) and patient survival in CCA (6). Interferons (IFNs) is well-known pro-inflammatory cytokines orchestrated in development of cancer including CCA. Additionally, MLKL expression was reported to be induced by interferons (IFNs)-mediated Stat1/IRF1 signaling during hepatic inflammation (69). In line, MLKL

expression was induced by IFNs/Stat1/IRF1 signaling in multiples cancer cells (70). Prediction analysis using the Eukaryotic Promoter Database confirmed that the promoter of MLKL contains IRF1 and Stat1 binding sites, indicating that Stat1/IRF1 might stimulate MLKL transcription by directly activating the MLKL promoter. Furthermore, MLKL was found to be up-regulated in a number of published datasets of IFN-regulated genes (70). Together, we therefore propose that MLKL upregulation might be regulated through epigenetic modification and IFNs/Stat1/IRF1 signaling in CCA tumor microenvironment. However, the molecular mechanisms underlying the regulation of MLKL expression in tumor cells have not been fully understood and need further study.

According to previous study, we have found that MLKL expression was positively correlated with the infiltration of TAMs, while its expression was negatively correlated with the infiltration of CD8 cytotoxic T cells (CD8+ T cells) which is anti-tumor immune cells (6). Therefore, these results suggested that MLKL might be associated with pro-inflammatory responses in CCA tumor microenvironment. Pro-inflammatory environment has been reported to promote cancer proliferation, invasion, and metastasis which are hallmarks of cancer (71). Therefore, in this study, in order to better understanding the protumorigenic roles of MLKL in CCA, we first analyzed the association between MLKL mRNA expression and gene signatures-associated with proliferation, epithelial-mesenchymal transition (EMT), migration and invasion obtained from the Gene Expression Omnibus (GEO) database. Of great interest, MLKL expression in CCA was positively correlated with several genes-associated with hallmarks of cancer mentioned above. These results support the pro-tumorigenic roles of MLKL in CCA. However, this correlation should be validated in different dataset which contain more larger sample size of patients. Other bioinformatic tools should be used in future studies to predict pathway analysis, transcription factors, promoter sequence to gain more knowledge about possible mechanism of MLKL in CCA.

Consistent with bioinformatics finding, we demonstrated that depletion of MLKL expression using short-hairpin RNA (shRNA) in CCA cells were significantly decreased in proliferation using MTT and colony formation assay. Moreover, the percentage of cells in S phase was significantly increased in MLKL knockdown CCA cells compared to shNT control, suggesting that MLKL might promote proliferation probably through regulation of cell cycle at S phase. Although there are several studies reported about the role of MLKL in cancer development and progression, its role on cancer proliferation and regulation in cell cycle phase have not been investigated. Therefore, this study might provide the new evidence supporting the effect of MLKL on cancer cell proliferation and cell cycle. However, these effects of MLKL and its underlying mechanisms are needed for further studies. Moreover, the specific method for cell proliferation (e.g. BrdU assay) and investigation the expression levels of major cell cycle-promoting factors should be required.

Regarding cell cycle regulation, it would be possible that MLKL might promote CCA proliferation probably through regulation of cell cycle at S phase. It is more likely that MLKL might locate and active in the nucleus to regulate cell cycle. Therefore, in order to understand MLKL localization, bioinformatics tool “cNLS Mapper” software was used to predict nuclear localization signals (NLSs) from amino acid sequence (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). Interestingly, we found that C-terminal of MLKL contains nuclear localization signals (NLSs) sequence at amino acids 224–256. Consistent with this finding, previous study reported that although majority of MLKL has been found in the cytoplasm, a small fraction of MLKL located in the nucleus in HUVEC endothelial cells to regulate gene expression (52). However, they found that MLKL could not directly bind DNA or RNA due to lacking DNA or RNA binding domain, but it interacts with other proteins such as an RNA-binding protein RBM6 to promote adhesion molecule expression by increasing mRNA stability through a non-necroptotic function (52). Altogether, we therefore propose that MLKL can translocate to the nucleus and might regulate the expression of cell cycle-related

factors by interact with other proteins, but not directly binding to DNA or RNA. Therefore, depletion of MLKL might dysregulate cell cycle transition, leading to slower transition of cells in S phase to G2/M phase, which results in a decrease in the proliferation of CCA cells. However, the molecular mechanisms by which MLKL regulate cell cycle and cell proliferation in CCA require further investigation.

Nowadays, necroptosis has been reported to contribute to cancer development and progression by promoting invasion and metastasis. For example, necroptosis induces proteolysis of E-cadherin and cell-cell contact in colorectal cancer cell lines, resulting in promoting tumor cell migration and invasion (72). In addition, necroptosis in endothelial cells can promote tumor cell extravasation and cancer metastasis (73). Nevertheless, key necroptotic factors including MLKL was reported to promote cancer invasion and EMT though a non-necroptotic function. For instance, depletion of MLKL expression inhibited invasion and increased epithelial markers (E-Cadherin) and decreased mesenchymal markers (Vimentin, N-Cadherin, Zeb1) of radioresistant nasopharyngeal carcinoma (NPC) cells (10). In addition, MLKL was highly expressed in hepatic stellate cells (HSCs) which contributes to hepatic fibrosis and that MLKL inhibition delays wound healing and vimentin expression in HSCs (9). These studies were consistent with our findings that MLKL might promote migration, invasion, and EMT in CCA cells. Although the expression of E-cadherin is not significantly changed, the expression of vimentin in MLKL knockdown cells is significantly decreased compared to shNT. These inconsistence expression between epithelial marker and mesenchymal marker have been reported in some previous studies. For example, trichostatin A (TSA) and valproic acid (VPA) increase not only the expression of E-cadherin but also increase in vimentin in CCA cell lines (74) which similar results were observed in human gastric and breast cancer cells (75). In addition, EMT which confers invasiveness and migratory properties to cells, is the initial step in carcinogenesis (76). Our CCA cell lines are invasive phenotype which EMT process has already happened.

Therefore, it might be the reason that EMT properties were not much changed in a resting condition compared to when stimulation with pro-inflammatory cytokines and E-cadherin, epithelial marker, was not change much in MLKL knockdown CCA cells. Altogether, it would be possible to imply that MLKL might promote EMT in CCA cells. However, our study investigated the role of MLKL on EMT only in mRNA level, which we cannot assume that the mRNA expression is correlated with their protein expression, further studies in protein levels are necessary. In addition, it is still unclear whether the protumorigenic role of MLKL in CCA is mediated through necroptosis or a non-necroptosis function, therefore, in order to discriminate whether the effects of MLKL on pro-tumorigenic properties is the results of necroptosis activation or its key molecules with a non-necroptotic function, inhibition of RIPK3 which is upstream regulator of MLKL in necroptosis signaling, should be required to validate these findings.

In conclusion, our finding is the first study to reveal that MLKL might have pro-tumorigenic role by promoting cell proliferation, EMT, migration and invasion in CCA cells. Apart from CCA development and progression, our preliminary studies obtained from Japanese non-Opisthorchis viverrini (Ov)-associated CCA cohort and our ongoing projects revealed that MLKL might induce immunosuppressive tumor microenvironment and/or therapeutic resistance in CCA. We found that high MLKL expression was significantly associated with both shorter disease-free survival (DFS) and overall survival (OS) and was positively correlated with a clinically unfavorable immune cell signature (high M2 TAMs and low CD8+ T cells) (6). Of great interest, our unpublished data also show a group of patients who received 5-fluorouracil (5-FU) adjuvant therapy with high MLKL expression had a shorter OS. These results suggest that MLKL might play a pro-tumorigenic role in CCA progression, immunosuppression, and 5-FU resistance, which require validation in future studies. (Figure 45). However, this finding is our preliminary *in vitro* results therefore further studies including mechanistic studies of how MLKL promote CCA development and progression and

validation in animal model are needed. Moreover, further studies should be required to clarify whether the effects of MLKL on pro-tumorigenic properties is the results of necroptosis activation or its key molecules with a non-necroptotic function. Finally, these finding provide that MLKL might be a promising therapeutic target for CCA targeted therapy.

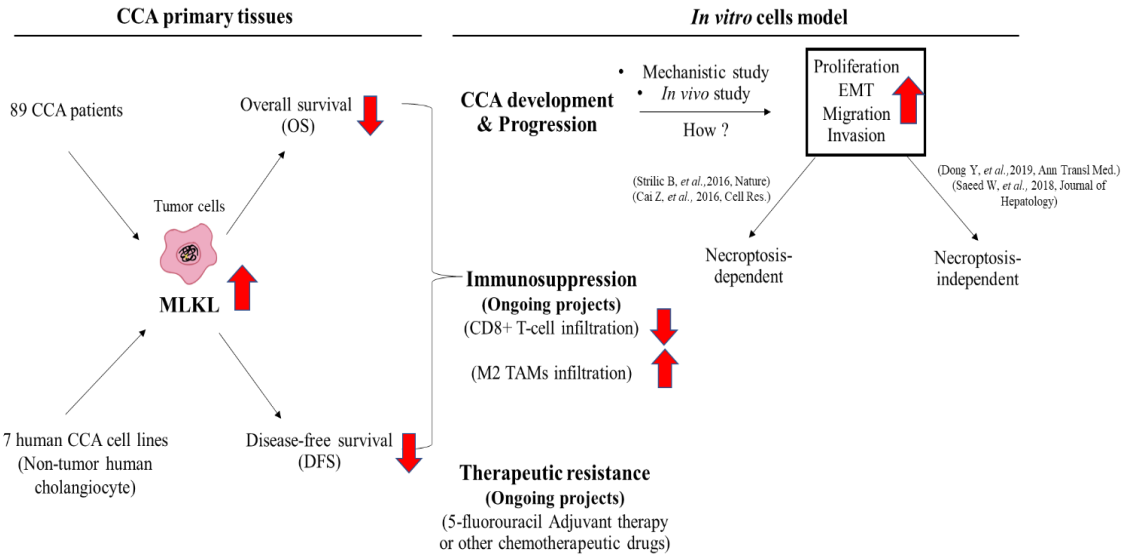

Figure 45 Schematic diagram of conclusions and future directions. The results presented in this study and ongoing studies related to MLKL novel functions are summarized and highlight future directions.

REFERENCES

1. Khan SA, Tavolari S, Brandi G. Cholangiocarcinoma: Epidemiology and risk factors. Liver Int. 2019;39 Suppl 1:19-31.

2. Jusakul A, Cutcutache I, Yong CH, Lim JQ, Huang MN, Padmanabhan N, et al. Whole-Genome and Epigenomic Landscapes of Etiologically Distinct Subtypes of Cholangiocarcinoma. Cancer Discov. 2017;7(10):1116-35.

3. Wu W, Liu P, Li J. Necroptosis: an emerging form of programmed cell death. Crit Rev Oncol Hematol. 2012;82(3):249-58.

4. Gong Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer. 2019;18(1):100.

5. Akara-Amornthum P, Lomphithak T, Choksi S, Tohtong R, Jitkaew S. Key necroptotic proteins are required for Smac mimetic-mediated sensitization of cholangiocarcinoma cells to TNF-alpha and chemotherapeutic gemcitabine-induced necroptosis. PLoS One. 2020;15(1):e0227454.

6. Lomphithak T, Akara-Amornthum P, Murakami K, Hashimoto M, Usubuchi H, Iwabuchi E, et al. Tumor necroptosis is correlated with a favorable immune cell signature and programmed death-ligand 1 expression in cholangiocarcinoma. Sci Rep. 2021;11(1):11743.

7. Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A. 2012;109(14):5322-7.

8. Park SY, Park HH, Park SY, Hong SM, Yoon S, Morgan MJ, et al. Reduction in MLKL-mediated endosomal trafficking enhances the TRAIL-DR4/5 signal to increase cancer cell death. Cell Death Dis. 2020;11(9):744.

9. Saeed W, Jun DW, Kim E, Kang BK, Jeong JY, Ahn SB, et al. Necroptosis signalling pathway in hepatic fibrosis; role of receptor-interacting serine-threonine kinase 3 and mixed lineage kinase domain-like in cirrhosis. . Journal of Hepatology

10. Dong Y, Sun Y, Huang Y, Fang X, Sun P, Dwarakanath B, et al. Depletion of MLKL inhibits invasion of radioresistant nasopharyngeal carcinoma cells by suppressing epithelial-mesenchymal transition. Ann Transl Med. 2019;7(23).

11. Colbert LE, Fisher SB, Hardy CW, Hall WA, Saka B, Shelton JW, et al. Pronecrotic mixed lineage kinase domain-like protein expression is a prognostic biomarker in patients with early-stage resected pancreatic adenocarcinoma. Cancer. 2013;119(17):3148-55.

12. He L, Peng K, Liu Y, Xiong J, Zhu FF. Low expression of mixed lineage kinase domain-like protein is associated with poor prognosis in ovarian cancer patients. Onco Targets Ther. 2013;6:1539-43.

13. Ruan J, Mei L, Zhu Q, Shi G, Wang H. Mixed lineage kinase domain-like protein is a prognostic biomarker for cervical squamous cell cancer. Int J Clin Exp Pathol. 2015;8(11):15035-8.

14. Ertao Z, Jianhui C, Kang W, Zhijun Y, Hui W, Chuangqi C, et al. Prognostic value of mixed lineage kinase domain-like protein expression in the survival of patients with gastric caner. Tumour Biol. 2016;37(10):13679-85.

15. Li X, Guo J, Ding AP, Qi WW, Zhang PH, Lv J, et al. Association of Mixed Lineage Kinase Domain-Like Protein Expression With Prognosis in Patients With Colon Cancer. Technol Cancer Res Treat. 2017;16(4):428-34.

16. Kambakamba P, DeOliveira ML. Perihilar cholangiocarcinoma: paradigms of surgical management. Am J Surg. 2014;208(4):563-70.

17. Srivatanakul P, Ohshima H, Khlat M, Parkin M, Sukaryodhin S, Brouet I, et al. Opisthorchis viverrini infestation and endogenous nitrosamines as risk factors for cholangiocarcinoma in Thailand. Int J Cancer. 1991;48(6):821-5.

18. Labib PL, Goodchild G, Pereira SP. Molecular Pathogenesis of Cholangiocarcinoma. BMC Cancer. 2019;19(1):185.

19. Squadroni M, Tondulli L, Gatta G, Mosconi S, Beretta G, Labianca R. Cholangiocarcinoma. Critical Reviews in Oncology/Hematology. 2016;116:11-31.

20. Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology. 2010;51(3):881-90.

21. Komori J, Marusawa H, Machimoto T, Endo Y, Kinoshita K, Kou T, et al. Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. Hepatology. 2008;47(3):888-96.

22. You Z, Bei L, Cheng LP, Cheng NS. Expression of COX-2 and VEGF-C in cholangiocarcinomas at different clinical and pathological stages. Genet Mol Res. 2015;14(2):6239-46.

23. Frampton G, Invernizzi P, Bernuzzi F, Pae HY, Quinn M, Horvat D, et al. Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut. 2012;61(2):268-77.

24. Smout MJ, Laha T, Mulvenna J, Sripa B, Suttiprapa S, Jones A, et al. A granulin-like growth factor secreted by the carcinogenic liver fluke, Opisthorchis viverrini, promotes proliferation of host cells. PLoS Pathog. 2009;5(10):e1000611.

25. Prakobwong S, Pinlaor P, Charoensuk L, Khoontawad J, Yongvanit P, Hiraku Y, et al. The liver fluke Opisthorchis viverrini expresses nitric oxide synthase but not gelatinases. Parasitol Int. 2012;61(1):112-7.

26. Jaiswal M, LaRusso NF, Shapiro RA, Billiar TR, Gores GJ. Nitric oxide-mediated inhibition of DNA repair potentiates oxidative DNA damage in cholangiocytes. Gastroenterology. 2001;120(1):190-9.

27. Sripa B, Brindley PJ, Mulvenna J, Laha T, Smout MJ, Mairiang E, et al. The tumorigenic liver fluke Opisthorchis viverrini--multiple pathways to cancer. Trends Parasitol. 2012;28(10):395-407.

28. Han J, Zhong CQ, Zhang DW. Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol. 2011;12(12):1143-9.

29. Dhuriya YK, Sharma D. Necroptosis: a regulated inflammatory mode of cell death. J Neuroinflammation. 2018;15(1):199.

30. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science. 2009;325(5938):332-6.

31. Moriwaki K, Chan FK. RIP3: a molecular switch for necrosis and inflammation. Genes Dev. 2013;27(15):1640-9.

32. Sun L, Wang H, Wang Z, He S, Chen S, Liao D, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell. 2012;148(1-2):213-27.

33. Arnez KH, Kindlova M, Bokil NJ, Murphy JM, Sweet MJ, Guncar G. Analysis of the N-terminal region of human MLKL, as well as two distinct MLKL isoforms, reveals new insights into necroptotic cell death. Biosci Rep. 2015;36(1):e00291.

34. Pujar S, O'Leary NA, Farrell CM, Loveland JE, Mudge JM, Wallin C, et al. Consensus coding sequence (CCDS) database: a standardized set of human and mouse protein-coding regions supported by expert curation. Nucleic Acids Res. 2018;46(D1):D221-d8.

35. Yuan J, Amin P, Ofengeim D. Necroptosis and RIPK1-mediated neuroinflammation in CNS diseases. Nat Rev Neurosci. 2019;20(1):19-33.

36. Iannielli A, Bido S, Folladori L, Segnali A, Cancellieri C, Maresca A, et al. Pharmacological Inhibition of Necroptosis Protects from Dopaminergic Neuronal Cell Death in Parkinson's Disease Models. Cell Rep. 2018;22(8):2066-79.

37. Ito Y, Ofengeim D, Najafov A, Das S, Saberi S, Li Y, et al. RIPK1 mediates axonal degeneration by promoting inflammation and necroptosis in ALS. Science. 2016;353(6299):603-8.

38. Ofengeim D, Ito Y, Najafov A, Zhang Y, Shan B, DeWitt JP, et al. Activation of necroptosis in multiple sclerosis. Cell Rep. 2015;10(11):1836-49.

39. Afonso MB, Rodrigues PM, Carvalho T, Caridade M, Borralho P, Cortez-Pinto H, et al. Necroptosis is a key pathogenic event in human and experimental murine models of non-alcoholic steatohepatitis. Clin Sci (Lond). 2015;129(8):721-39.

40. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology. 2014;147(4):765-83.e4.

41. Lin J, Li H, Yang M, Ren J, Huang Z, Han F, et al. A role of RIP3-mediated macrophage necrosis in atherosclerosis development. Cell Rep. 2013;3(1):200-10.

42. Wang Q, Liu Z, Ren J, Morgan S, Assa C, Liu B. Receptor-interacting protein kinase 3 contributes to abdominal aortic aneurysms via smooth muscle cell necrosis and inflammation. Circ Res. 2015;116(4):600-11.

43. Bozec D, Iuga AC, Roda G, Dahan S, Yeretssian G. Critical function of the necroptosis adaptor RIPK3 in protecting from intestinal tumorigenesis. Oncotarget. 2016;7(29):46384-400.

44. Stoll G, Ma Y, Yang H, Kepp O, Zitvogel L, Kroemer G. Pro-necrotic molecules impact local immunosurveillance in human breast cancer. Oncoimmunology. 2017;6(4):e1299302.

45. Zhao Q, Yu X, Li M, Liu Y, Han Y, Zhang X, et al. MLKL attenuates colon inflammation and colitis-tumorigenesis via suppression of inflammatory responses. Cancer Lett. 2019;459:100-11.

46. Liu X, Zhou M, Mei L, Ruan J, Hu Q, Peng J, et al. Key roles of necroptotic factors in promoting tumor growth. Oncotarget. 2016;7(16):22219-33.

47. Ando Y, Ohuchida K, Otsubo Y, Kibe S, Takesue S, Abe T, et al. Necroptosis in pancreatic cancer promotes cancer cell migration and invasion by release of CXCL5. PLoS One. 2020;15(1):e0228015.

48. Seifert L, Werba G, Tiwari S, Giao Ly NN, Alothman S, Alqunaibit D, et al. The necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. Nature. 2016;532(7598):245-9.

49. Shibata K, Omahdi Z, Yamasaki S. Necroptosis DAMPens anti-tumor immunity. Cell Death Discov. 2016;2:16033.

50. Ying Z, Pan C, Shao T, Liu L, Li L, Guo D, et al. Mixed Lineage Kinase Domain-like Protein MLKL Breaks Down Myelin following Nerve Injury. Mol Cell. 2018;72(3):457-68.e5.

51. Yoon S, Kovalenko A, Bogdanov K, Wallach D. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. Immunity. 2017;47(1):51-65.e7.

52. Dai J, Zhang C, Guo L, He H, Jiang K, Huang Y, et al. A necroptotic-independent function of MLKL in regulating endothelial cell adhesion molecule expression. Cell Death Dis. 2020;11(4):282.

53. Koo GB, Morgan MJ, Lee DG, Kim WJ, Yoon JH, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell Res. 2015;25(6):707-25.

54. Feng X, Song Q, Yu A, Tang H, Peng Z, Wang X. Receptor-interacting protein kinase 3 is a predictor of survival and plays a tumor suppressive role in colorectal cancer. Neoplasma. 2015;62(4):592-601.

55. Hockendorf U, Yabal M, Herold T, Munkhbaatar E, Rott S, Jilg S, et al. RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. Cancer Cell. 2016;30(1):75-91.

56. Nugues AL, El Bouazzati H, Hetuin D, Berthon C, Loyens A, Bertrand E, et al. RIP3 is downregulated in human myeloid leukemia cells and modulates apoptosis and caspase-mediated p65/RelA cleavage. Cell Death Dis. 2014;5:e1384.

57. Kim J, Chung JY, Park YS, Jang SJ, Kim HR, Choi CM, et al. Prognostic Significance of CHIP and RIPK3 in Non-Small Cell Lung Cancer. Cancers (Basel). 2020;12(6).

58. Dong Y, Sun Y, Huang Y, Dwarakanath B, Kong L, Lu JJ. Upregulated necroptosis-pathway-associated genes are unfavorable prognostic markers in low-grade glioma and glioblastoma multiforme. Transl Cancer Res. 2019;8(3):821-7.

59. Yamauchi T, Fujishima F, Hashimoto M, Tsunokake J, Akaishi R, Gokon Y, et al. Necroptosis in Esophageal Squamous Cell Carcinoma: An Independent Prognostic Factor and Its Correlation with Tumor-Infiltrating Lymphocytes. Cancers (Basel). 2021;13(17).

60. Li J, Huang S, Zeng L, Li K, Yang L, Gao S, et al. Necroptosis in head and neck squamous cell carcinoma: characterization of clinicopathological relevance and in vitro cell model. Cell Death Dis. 2020;11(5):391.

61. Morgan MJ, Kim YS. The serine threonine kinase RIP3: lost and found. BMB Rep. 2015;48(6):303-12.

62. Moriwaki K, Bertin J, Gough PJ, Orlowski GM, Chan FK. Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. Cell Death Dis. 2015;6:e1636.

63. Najafov A, Zervantonakis IK, Mookhtiar AK, Greninger P, March RJ, Egan RK, et al. BRAF and AXL oncogenes drive RIPK3 expression loss in cancer. PLoS Biol. 2018;16(8):e2005756.

64. Sun W, Yu W, Shen L, Huang T. MLKL is a potential prognostic marker in gastric cancer. Oncol Lett. 2019;18(4):3830-6.

65. Rattanasinganchan P, Leelawat K, Treepongkaruna SA, Tocharoentanaphol C, Subwongcharoen S, Suthiphongchai T, et al. Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient. World J Gastroenterol. 2006;12(40):6500-6.

66. Miyagiwa M, Ichida T, Tokiwa T, Sato J, Sasaki H. A new human cholangiocellular carcinoma cell line (HuCC-T1) producing carbohydrate antigen 19/9 in serum-free medium. In Vitro Cell Dev Biol. 1989;25(6):503-10.

67. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-8.

68. Gong Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer. 2019;18.

69. Günther C, He GW, Kremer AE, Murphy JM, Petrie EJ, Amann K, et al. The pseudokinase MLKL mediates programmed hepatocellular necrosis independently of RIPK3 during hepatitis. J Clin Invest. 2016;126(11):4346-60.

70. Knuth AK, Rösler S, Schenk B, Kowald L, van Wijk SJL, Fulda S. Interferons Transcriptionally Up-Regulate MLKL Expression in Cancer Cells. Neoplasia. 2019;21(1):74-81.

71. Singh N, Baby D, Rajguru JP, Patil PB, Thakkannavar SS, Pujari VB. Inflammation and Cancer. Ann Afr Med. 2019;18(3):121-6.

72. Cai Z, Zhang A, Choksi S, Li W, Li T, Zhang XM, et al. Activation of cell-surface proteases promotes necroptosis, inflammation and cell migration. Cell Res. 2016;26(8):886-900.

73. Strilic B, Yang L, Albarrán-Juárez J, Wachsmuth L, Han K, Müller UC, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. Nature. 2016;536(7615):215-8.

74. Wang JH, Lee EJ, Ji M, Park SM. HDAC inhibitors, trichostatin A and valproic acid, increase E-cadherin and vimentin expression but inhibit migration and invasion of cholangiocarcinoma cells. Oncol Rep. 2018;40(1):346-54.

75. Han RF, Li K, Yang ZS, Chen ZG, Yang WC. Trichostatin A induces mesenchymal-like morphological change and gene expression but inhibits migration and colony formation in human cancer cells. Mol Med Rep. 2014;10(6):3211-6.

76. Yao X, Wang X, Wang Z, Dai L, Zhang G, Yan Q, et al. Clinicopathological and prognostic significance of epithelial mesenchymal transition-related protein expression in intrahepatic cholangiocarcinoma. Onco Targets Ther. 2012;5:255-61.

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