POST-DOCTORAL FELLOWSHIP

Principal Investigator: Dr. Salvatore Papa (Head of laboratory and applicant)
Named scientist: Gemma Choy (member of the group)

1. – TITLE OF THE RESEARCH

MAPK-targeted therapy for intrahepatic cholangiocarcinoma

2. – SUMMARY

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy after hepatocellular carcinoma, accounting for approximately 10-15% of primary liver cancer [13]. Rising trends in the incidence of ICC have been well recognized worldwide [1-3]. Although effective new treatments have increased survival for many other cancers during the past 30 years, treatment strategies and survival for patients with ICC have improved little [1-3]. Still worse, ICC patients respond poorly to aggressive chemotherapy or radiotherapy [4]. Thus, identifying effective treatment strategies to improve outcome is one of the major challenges and future needs for ICC.

Recent experimental evidence obtained in our laboratory suggests that the c-Jun N-terminal kinase (JNK) pathway is up-regulated in human intrahepatic cholangiocarcinoma and serves as a potential therapeutic target. In this on-going research we plan to test whether inhibition of the JNK proteins and its downstream effectors enhances the sensitization of ICC cells to current chemotherapeutic drugs.

3. – BACKGROUND AND AIM OF THE PROJECT

Cancer cells depend on oncogenic signals to promote cell cycle progression and prevent cell death (or apoptosis) that would otherwise result from aberrant stress [5]. The JNK pathway is one of the major signalling components of the mitogen-activated protein kinase (MAPK) signalling pathway that is constitutively activated by oncogenic signals. JNK functions in the control of a number of cellular processes, including proliferation, embryonic development and apoptosis by controlling the activation of many transcription factors and downstream effectors (**Figure 1**) [6]. The JNK pathway is activated by an intricate number of mechanisms, one of these is through activation of GTP-binding proteins such as RAS. Genetic point mutations activating the RAS protein and its downstream JNK cascade have been observed in many human tumours [6,7]. Targeting JNK cascade has thus become an attractive therapeutic strategy in human cancers, including liver cancer [6]. However, whether the JNK cascade is involved in primary human ICC is not known. We are the first group to investigate the function of JNK signalling in this deleterious human condition. Using a cohort of ICC patients, we found that the protein kinase JNK was highly active in ICC and its activity correlates with expression of downstream cell cycle regulatory proteins, such as peptidyl-prolyl-isomerase PIN1 and cyclin D1. Depletion of either JNK or PIN1 in a panel of ICC cells significantly reduces cancer cell proliferation and tumor growth *in vitro*. Current analyses are also exploring whether depletion of JNK and PIN1 affect the growth of animal-implanted tumors (xenografeted tumors) to further support the hypothesis that the JNK/PIN1 axis is important for tumor cell growth both *in vitro* and *in vivo*.

In the proposed study, we aim to test the preclinical activities of the JNK inhibitor, JNK-IN-8, and PIN1 inhibitor, PiB, against ICC cells [8,9].

4. – WORK LEADING TO THE PROPOSED PROJECT

To address the activation status of JNK protein kinase in human ICC, the expression of phospho-JNK was determined by immunohistochemical staining in tumor samples from a cohort of 14 ICC patients. The results showed that, as compared with intrahepatic bile duct cells that showed weak or negative phosphorylated JNK, high expression of phospho-JNK was observed in 71.4% (10/14) of ICC cases (**Figure 2**). Likewise, expression of PIN1, a downstream effector of the JNK cascade, was scored as high intensity in 64.3% (9/14) (**Figure 2**). These data indicated that JNK-PIN1 pathway was upregulated in almost two third of human ICC. Strikingly, elevated expression of PIN1 transcripts was also observed in two independent cohorts of human ICC compared to their normal liver tissue. We found that, the expression of PIN1 mRNA was significantly higher than that in normal biliary epithelial cells (**Figure 3A**). Likewise, we also observed significantly higher expression of PIN1 in ICC samples compared to their adjacent nontumor tissues (**Figure 3B**). Consistent with the *in-vivo* analyses of human samples, immunoblotting analyses showed constitutive activation of JNK and expression of PIN1 in ICC-derived cell lines, but not in nontumoral immortalised H69 cholangiocytes (**Figure 4**). These analyses indicate that a strong relationship between JNK activation and PIN1 expression exists only in cancer ICC cells, but not in normal liver cells, including intrahepatic bile-duct cells. Altogether our data suggest that either JNK or PIN1 may represent a target for ICC treatment.

With the support of a **Post-Doctoral Fellowship** from the *Alan Morement Memorial Fund*, **we now propose to test whether inhibition of either JNK or PIN1 will effectively enhance the death of cancer cells in combination with current chemotherapy drugs**, such as 5-FU, cisplatin, gemcitabine or doxorubicin [4]. These experiments will be performed both *in vitro*, using human ICC-derived cell lines, and *in vivo*, using tumor-implanted animals. These preclinical studies will be fundamental to determine whether JNK and PIN1 inhibition can be used as new (or adjuvant) therapeutic approaches to current therapies [4].

5. – PLAN OF INVESTIGATION

Methodology

Use of chemical inhibitors to inhibit the enzymatic activity of either JNK or PIN1 – JNK-IN-8 is a selective JNK inhibitor that inhibits phosphorylation of c-Jun (a direct substrate of JNK), in cells exposed to submicromolar drug in a manner that depends on covalent modification of the conserved cysteine residue [8]. PiB is an ester-containing symmetrical compound reported to have an inhibitory effect on the enzymatic activity of PIN1, which catalyse the isomerization of the Ser(Thr)-Pro bond from *cis* to *trans*, altering the conformation and the biological function of target proteins.

Use of shRNA system to knockdown either JNK or PIN1 – As already established in the laboratory, we would also use shRNA-mediated knockdown of JNK and PIN1 to deplete the expression of these proteins in cancer cells. This is a very powerful technique that allows studying the functional role of a certain protein in tumors.

Experimental Plan

In vitro experiments using JNK and PIN1 chemical inhibitors – To determine the growth inhibition effect of anti-ICC drugs (i.e. cisplatin, gemcitabine, 5-FU) in combination with JNK chemical inhibitor (JNK-IN8), human ICC-derived cells SG231, HuCCT1 and CCLP-1 will be incubated for 72 hours in presence of increasing doses of JNK-IN8 and/or cisplatin and cell proliferation and apoptosis will be assessed using proliferation and apoptosis assay. Likewise, the combinatorial effect of anti-ICC drugs and PIN1 chemical inhibitor (PiB) will be also assessed. In order to confirm the specificity of these two chemical inhibitors in ICC cells, JNK kinase assay and PIN1 enzymatic activity will be performed in a dose-dependent treatment of ICC-derived cells.

In vitro experiments using JNK-depleted and PIN1-depleted cells – To evaluate whether suppression of JNK-PIN1 signalling have an additive effect on cell death induced by current chemotherapeutic drugs, we aim to silence or overexpress PIN1 in ICC cells and their viability will be assayed after treatment with

cisplatin. Finally, we will apply these analyse to other clinically relevant compounds used for the treatment of ICC, including 5-FU, doxorubicin.

In vivo animal experiments – To investigate the effects of JNK/PIN1 inhibition on the growth of ICC xenografts, we will monitor whether cisplatin (or any other clinically relevant anti-ICC treatment, i.e. 5-FU, gemcitabine, doxorubicin) acts synergistically with either JNK or PIN1 inhibition to promote apoptosis in ICC cells. Immune-suppressed NOD/SCID mice will be subcutaneously injected with ICC cells bearing JNK, PIN1 or control (NS) shRNAs. Xenografted mice (14 per group) will be administered intra-peritoneum either with 2 mg/kg cisplatin or equivalent saline for 10 days (5 days *on* + 5 days *off*, for 2 cycles) and growth of established xenografts will be monitored at least twice weekly, and tumor volume will be measured. Animals will be sacrificed on day 18 during the treatment, body weight and tumor weights will be recorded, and tumors will be harvested for analysis. To examine the antiproliferative and apoptotic effects of JNK/PIN1 inhibition plus cisplatin, in vivo, sections from treated tumors will be stained with Ki-67 and cleaved caspase-3 antibodies.

With this study we will provide evidence that either JNK or PIN1 inhibition can be combined with other anti-ICC treatment to increase cytotoxicity when compared with either inhibition alone. This observation will have a major clinical implication because JNK/PIN1 inhibition may not be optimally effective as a monotherapy in ICC tumors. Therefore, the use of JNK/PIN1 inhibitors combined with anti-ICC drugs may have great value as an alternative approach in the treatment of this fatal disease.

References:

1. Khan SA, Emadossadaty S, Ladep NG, Thomas HC, Elliott P, Taylor-Robinson SD, Toledano MB. (2012). Rising trends in cholangiocarcinoma: Is the ICD classification system misleading us? **J Hepatol**. 56: 848854.

2. Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. (2014). Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma**. J Hepatol.** 60(6):1268-1289.

3. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. (2002). Changing international trends in mortality rates for liver, biliary and pancreatic tumours. **J Hepatol** 37: 806–813.

4. Rizvi S, Gores GJ. (2013). Pathogenesis, diagnosis, and management of cholangiocarcinoma. **Gastroenterology** 145(6): 1215-1229.

5. Hanahan D, Weinberg RA. (2011). Hallmarks of cancer: the next generation. **Cell**. 144: 646-674.

6. Bubici C, Papa S. (2014). JNK signalling in cancer: in need of new, smarter therapeutic targets. **Br J Pharmacol**. 171: 24-37.

7. Zhu AX, Borger DR, Kim Y, Cosgrove D, Ejaz A, Alexandrescu S, Groeschl RT, Deshpande V, Lindberg JM, Ferrone C, Sempoux C, Yau T, Poon R, Popescu I, Bauer TW, Gamblin TC, Gigot JF, Anders RA, Pawlik TM. (2014) Genomic Profiling of Intrahepatic Cholangiocarcinoma: Refining Prognosis and Identifying Therapeutic Targets. **Ann Surg Oncol**. [Epub ahead of print]

8. Zhang T, Inesta-Vaquera F, Niepel M, Zhang J, Ficarro SB, Machleidt T, Xie T, Marto JA, Kim N, Sim T, Laughlin JD, Park H, LoGrasso PV, Patricelli M, Nomanbhoy TK, Sorger PK, Alessi DR, Gray NS. (2012). Discovery of potent and selective covalent inhibitors of JNK. **Chem Biol**. 19: 140-154.

9. Moore JD, Potter A. (2013). Pin1 inhibitors: Pitfalls, progress and cellular pharmacology. **Bioorg Med Chem Lett**. 23(15): 4283-4291

6. – SUPPORTING FIGURES

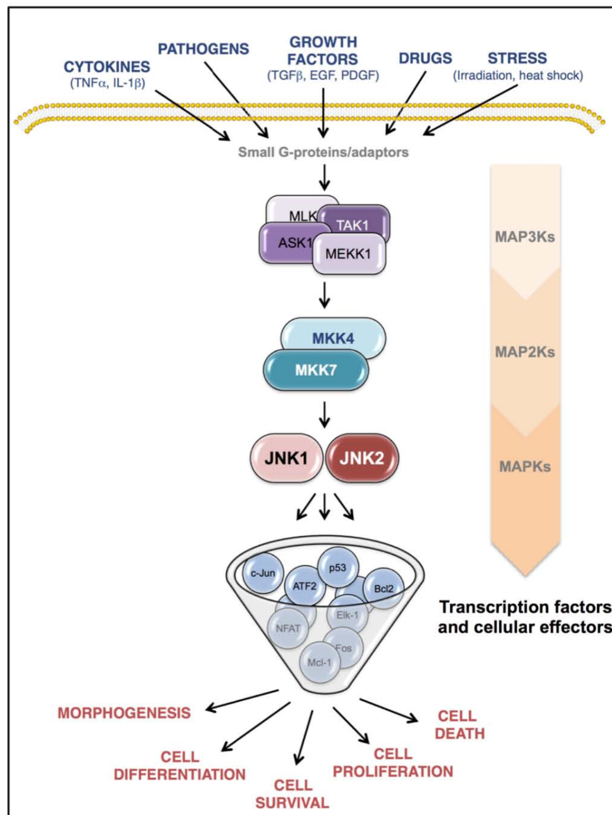

FIGURE 1. Schematic representation of the JNK signalling cascade *(adapted from Bubici C. and Papa S., Br J Pharmacol 2014)***.** Like all other MAPK, the JNK signalling cascade consists of a three-component module of upstream MAP3Ks that couples the signals from the cell surface to intracellular protein effectors. Different MAP3Ks have been implicated in the JNK cascade, depending on the type of stimulus the cell receives. Once activated, MAP3Ks phosphorylate and activate components of the MAP2K module, which in turn phosphorylate and stimulate the activity of distinct JNK isoforms. Upon activation, each JNK protein itself can phosphorylate serine and threonine residues on specific substrates, delivering different cellular activities.

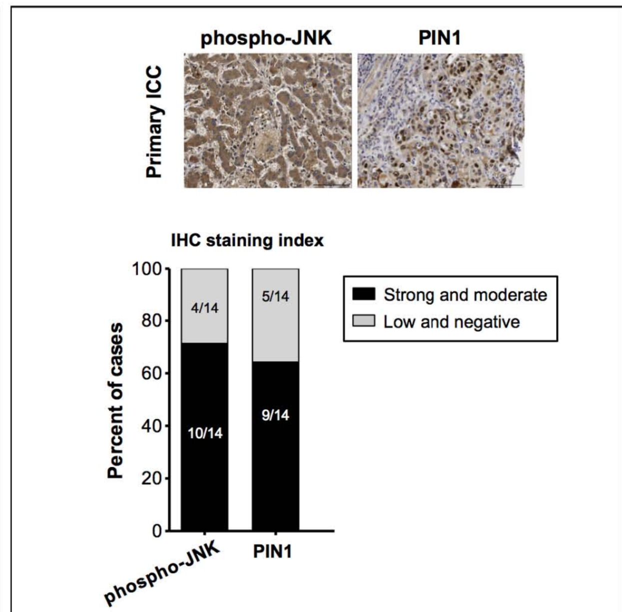

FIGURE 2. Expression of phospho-active JNK and PIN1 in human ICC biopsies. Phospho-JNK and PIN1 immunostaining of tissue microarray comprising 14 ICC livers. Shown are representative images of the immunostainings at 20X magnification. Graph indicates the percentage of cases displaying strong or low staining intensity of either phospho-JNK or PIN1.

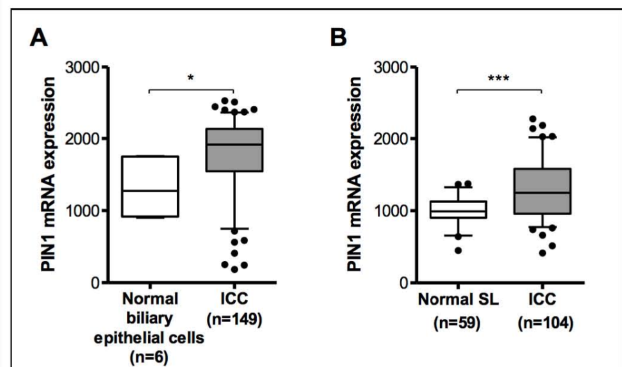

FIGURE 3. Expression of PIN1 in human ICC biopsies. Gene-expression analysis of PIN1 transcripts in ICC patients from the NCBI GEO database showing significantly higher expression of PIN1 in tumor tissues compared to normal biliary epitheleial cells (A) or surrounding non-tumor liver tissues (B). For each box plot, the whiskers represent the 2.5-97.5th percentile range of values, the lower and up boundaries denote the 25th and 75th percentile of each data set, respectively, and the horizontal line represents the median value for each group. P values were calculated by nonparametric Mann-Whitney test. *$P<0.05$; ***$P<0.0001$

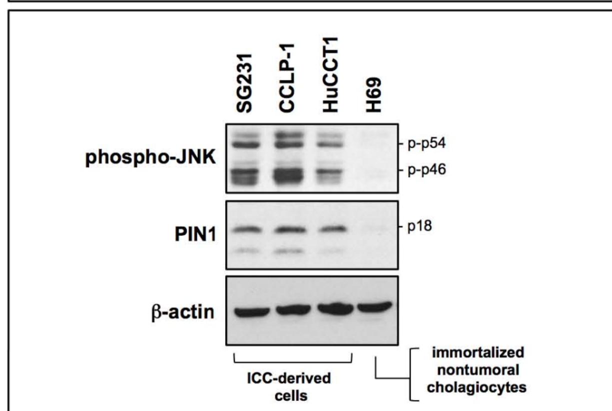

FIGURE 4. Expression of phospho-active JNK and PIN1 in human ICC biopsies. Protein analyses showing elevated expression of phospho-JNK and PIN1 in ICC-derived cells compared to nontumoral cholangiocytes. β-actin expression was used as loading control.

2-year Fellowship – Dr Gemma Choy

The experiments in this research integrate cancer biology with biochemistry, molecular biology and mouse molecular genetics. Therefore they require people that have been trained in a multi-disciplinary fashion and to a high degree of competence. Post-doctoral fellow, Pui Man (Gemma) Choy, because of her higher level of training, is best suited for the work proposed.

Gemma has spent three years in Dr Salvatore Papa's laboratory as PhD student and has submitted successfully her final thesis. She discussed her Viva in December 2014, and has been awarded her PhD.

As a testament of her capabilities, she was named a second author of a recent study in hepatocellular carcinoma under review at *Nature Communication (2013 impact factor: 10.742).*

Gemma will be responsible for examining the role of JNK and PIN1 in ICC carcinogenesis. Under the direct supervision of the Principal Investigator, Dr Salvatore Papa, she will apply cellular and molecular biology techniques to the analysis of *in vitro* and *in vivo* models to address the questions outlined in the proposed research.