2. GH measurement

Blood sample of 200-300 µl of were drawn from mouse via the saphenous vein and collected in a tube containing 10 µl of 50 mg/ml EDTA and gently mixed to prevent blood clotting. Then the samples were centrifuged at 1,600 g for 20 min to separate plasma and packed cells. Plasma can be kept at -20 °C until further assay.

To measure plasma growth hormone level in mice, rat growth hormone enzyme linked immunoassay can be used because the anti-growth hormone antibody in this system has 91% cross reactivity to mouse anti-growth hormone antibody. The concept of this method is the competitive binding of rat anti-growth hormone antibody and its derivative, anti-growth hormone antibody conjugated with acethylcholine esterase, with free growth hormone in the plasma. The more free plasma growth hormone, the more the signal from acethylcholine esterase can be measured. The OD414 of each sample was measured and quantitated by comparing with the standard curve.

3. Quantitative real time-reverse transcriptase-polymerase chain reaction (RT-PCR)

Mouse liver tissue of 200-250 mg was cut and total RNA was extracted by the RNeasy kit (Qiagen). The quality of RNA was checked by measuring at OD260/280. Five µg of total RNA was converted to cDNA using oligo dT primer and Superscript II, reverse transcriptase. For real time RT-PCR, the primers for each interesting genes (*egfr*, *cxbl*, *igf1*, *cyp2a*, *cyp4a14* and *msta2*) were designed using a computer program available in the ABI 7700 machine. The volume of cDNA equal to 250 ng was used in SYBR real time RT-PCR running in the ABI 7700. The quantity of each interesting gene was performed comparing this gene to that of the internal control genes, *β-actin* or *gapdh*. The real time RT-PCR of all the genes listed above was performed in liver tissues from B6 and B6-*lit/lit* mice.

4. Somatostatin analogue toxicity test

Forty of each B6 and B6-Hcs7^{C3H} strains were used in the study of the sandostatin analogue toxicity. The optimal concentration tested in rat, 10-30 mg/kg, will be primarily used. The desired drug amount will be administered into the mice and 7 days after that the well being of mice will be observed.

5. Pharmacokinetic study of somatostatin analogue

Twenty B6 and B6-Hcs7^{C3H} mice at 6-week old will be used by dividing them into 2 groups. The first group will be for the drug test and the other group will be the control group without any drug treatment. After administration of somatostatin analogue for 1, 5, 10, 20, 30, 40, 50 and 60 d, blood will be collected for measuring the plasma growth hormone level. Triplicate

experiment will be done to get the value with statistical significance. The final value of growth hormone level will be considered to determine the best drug administration time to have the peak level of drug in mice.

6. Effect of somatostatin analogue in mouse hepatocarcinogenesis

The effect of somatostatin analogue in mouse hepatocarcinogenesis will be assessed in 290 of each B6 and B6-Hcs7^{C3H} and 40 of B6-*lit/lit* and B6-Hcs7^{C3H}-*lit/lit* male mice. After the drug dose and kinetics have been established, the somatostatin analogue will be used to treat B6 and B6-Hcs7^{C3H} mice divided into five groups. All mice will be treated at 12 days of age with DEN to initiate hepatocarcinogenesis. Three experimental groups will be used to determine somatostatin analogue's ability to treat liver carcinogenesis when given before, during or after tumor development. Two control groups, one wild type and one growth hormone deficient (*lit/lit*), will not receive drug treatment. Groups one, two and three will begin treatment with the somatostatin analogue at 2 weeks, 20 weeks and 32 weeks of age, respectively. The dose will be administered every 4 weeks until 44 weeks of age. Groups of 10 mice will be sacrified every four weeks beginning at 20 weeks (group 1, 2 and untreated controls) or 32 weeks (group 3), the final groups will be sacrified at 48 weeks. The numbers of liver tumors and preneoplastic foci in the group of animals treated with the somatostatin analogue are the major endpoints. The efficiency of somatostatin analogue will be judged by its ability to reduce the numbers of tumors or preneoplastic liver tumor foci that develop in carcinogen-treated mice. At each time point, blood will be collected for growth hormone measurement and liver tissues will be collected for QRT-PCR and for analysis of preneoplastic lesions. The Wilcoxon rank sum test will be used to determine the statistical significance of differences in tumor development between the somatostatin analogue treated and untreated groups (10).

RESULTS

1. Mouse genotyping

More than 300 mice were tested at the DNA level for their genotypes. The genotyping of B6, C3H and their heterozygous, B6-Hcs7^{C3H}, was performed by DNA polymorphism. The different patterns of bands of each strain allowed us to distinguish each individual strain. The genotyping of the each wild type and its derivative, *lit/lit*, was performed based on the different recognition sites on the PCR fragment of *ghrhr* gene of these 2 strains. There were a collection of mice ready for doing experiments including, pharmacokinetic and somatostatin analogue

toxicity tests. The summarization of numbers, sex and the strains of mice is shown in the following table,

Table 1 Summarization of number and age of B6-Hcs7^{C3H} male and female mice

Strain (sex)	DEN treated	No. of mice	For the study of
B6-Hcs7^{C3H} (M)	No	36	Pharmacokinetic and toxicity test
B6-Hcs7^{C3H}-*lit/lit* (M)	No	15	Control
B6-Hcs7^{C3H} (M)	Yes	11	Effect of somatostatin analogue in mouse hepatocarcinogenesis
B6-Hcs7^{C3H}-*lit/lit* (M)	Yes	8	Effect of somatostatin analogue in mouse hepatocarcinogenesis
B6-Hcs7^{C3H} (F)	No	28	Stock for breeding
B6-Hcs7^{C3H}-*lit/lit* (F)	No	10	Stock for breeding

2. Measurement of mouse plasma growth hormone

The growth hormone level in mice at different time of blood collection was studied because the secretion of this hormone depends on time and activity of the mice. The blood collection time ranged from 10 am to 1 pm. The level of growth hormone was measured in B6 male and female mice to find the growth hormone profiles in each sex. The result is shown in the following figures.

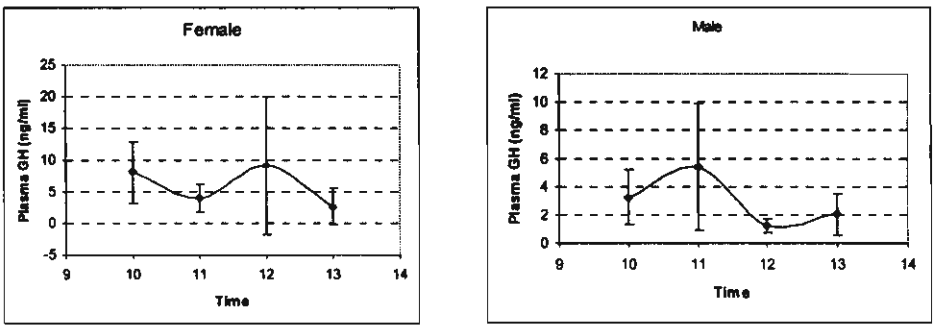

Figure 1 Profile of growth hormone secretion in male and female B6 mice

3. Real time RT-PCR

The total RNA was extracted from liver tissues of wild type B6 and B6-*lit/lit* male mice. Real time RT-PCR of *egfr*, *cxbl*, *igf1*, *cyp2a*, *cyp4a14* and *msta2* genes was performed. The

result can be used to classify genes into 2 groups depending on their levels of expression in B6-*lit/lit* compared to that of B6 mouse (Table 2 and Table 3). The first group is the downregulated genes in B6-lit/lit, i.e. *egfr*, *cxbl*, and *igf1*, the other group is upregulated genes in B6-*lit/lit*, i.e. *cyp2a*, *cyp4a14* and *msta2*. These results are comparable to those from Bugni et al (10).

Table 2 The down-regulated genes in B6-*lit/lit* mouse

Gene name	B6 male	B6-*lit* male	*B6*-lit/lit male (Bugni et al.) (10)
EGFR	1	0.021	0.058±0.014
Cxbl	1	0.028	0.14±0.034
Igf1	1	0.064	0.21±0.044

Note : using *β-actin* as an internal control

Table 3 The up-regulated genes in B6-*lit/lit* mouse

Gene name	B6 male	B6-*lit* male	*B6*-lit/lit male (Bugni et al.) (10)
Cyp2a	1	5.08	15±3.2
Cyp4a14	1	4.45	4.2±1.4
Msta2	1	2.8×10^5	370±78

Note : using *gapdh* as an internal control

DISCUSSION

Hepatocellular carcinoma is the eighth leading cause of cancer-related deaths in the world (1). The studies in both human and animal indicate that growth hormone can increase incidence of this cancer. Many anticancer drugs have been tried to apply in the hepatocellular carcinoma patients. The somatostatin analogue, a synthetic analogues of the natural hormone, somatostatin, is one of the drug that gives a good result in the decrease of growth hormone level in human. So far, there are many clinical trials that have attempted to use this drug in the treatment of hepatocellular carcinoma patients. Our collaborators in University of Wisconsin Comprehensive Cancer Center (UWCCC) designed the study to test the effect of somatostatin analogue in liver tumor patients especially on growth hormone-related gene expression in treats and untreated tumors. These studies will establish whether any effects of the somatostatin analogue on liver tumors could be explained by the suppression of the growth hormone pathway.

We propose to complement these human studies with more manipulable mouse studies. Specifically, we will test the somatostatin analogue's effectiveness in both preventing and treating liver cancer in carcinogen-treated mice of strains that differ in their sensitivity to liver cancer.

The effect of somatostatin analogue in mouse hepatocarcinogenesis will be assessed in B6 and B6-Hcs7^{C3H}mice while B6-*lit/lit* and B6-Hcs7^{C3H}-*lit/lit* mice will be used as the controls. In order to breed the enough numbers of mice needed in the experiment, a certain time waiting for the breeding process was needed. Meanwhile, the mouse genotyping was performed to get rid the unwanted strains. During one year of doing postdoctoral training, a collection of wanted mice was obtained as listed in Table 1. They are avialable to perform pharmacokinetic and drug toxicity tests. Even more mice were needed to be involved in study the effect of the somatostatin analogue in mouse hepatocarcinogenesis. Female mice of both B6-Hcs7^{C3H} and B6-Hcs7^{C3H}-*lit/lit* were needed for further breeding. The measurement of growth hormone level in the mouse plasma was performed using the enzyme immunoassay technique. Though the antibody against growth hormone in this system is specifically for rat, it has 91% of cross-reactivity to mouse. The profile of growth hormone secretion indicated the peak of plasma growth hormone level during 10 to 11 am in male mice. The plasma growth hormone level in female mice seems to be constant during this time period. It can be explained by the theoretical reason that male mice usually have a several peaks of growth hormone level in a day whereas in female only one peak of growth hormone level is detected. The level of plasma growth hormone in female is constant through the day. These preliminary data suggested that in the study of the effect of the somatostatin analogue in mouse hepatocarcinogenesis, mice should be breeded at around 10-11 in the morning for measurement of plasma growth hormone level.

The expressions of growth hormone-related genes, *egfr*, *cxbl*, and *igf1* genes decreased whereas the expressions of *cyp2a*, *cyp4a14* and *msta2* genes increased in mice with naturally low levels of growth hormone, B6-*lit/lit* (Table 2 and 3). The *lit/lit* mouse has been proved that it has a lower incidence of liver tumor formation (10). It might then be concluded that a low level of growth hormone may decrease liver tumor carcinogenesis by controlling at the expression of growth hormone-related genes. This study to answer whether the effect of somatostatin analogue can decrease hepatocarcinogenesis by altering these gene expressions will be further performed.

FURTHER WORKS

1. The somatostatin analogue toxicity and pharmacokinetic of this drug are being performed as described in the Materials and Methods.

2. The productions of B6-Hcs7^{C3H} congenic mice and B6-Hcs7^{C3H}-*lit/lit* have been partially produced. After having the designed number of mice, effect of the somatostatin analogue against mouse hepatocarcinogenesis will be studied using the method established in the preliminary experiment.

THE APPLICABLE ADVANTAGES

I received a great experience in both research and daily living during my 1-year postdoctoral training in the McArdle Lab for Cancer Research, University of Wisconsin-Madison, USA. The opportunity to know and work with a famous researcher such as Prof. Norman Drinkwater, a director of McArdle Laboratories and my mentor as well, encourage me to make a future collaboration with him and his colleagues. As I mentioned in the 6-month progress report that even though my postdoctoral project in McArdle Lab does not directly impact my work in Thailand, I learned to know how to study the carcinogenesis in mice using liver cancer as a model. This will be applied to explore cholangiocarcinogenesis in the future. For the research outcome, I can't accomplish this entire project by myself since it requires a longer time to do the whole project. I have the chance to learn from my mentor the progression of this project. He and his research assistant will continue this project. The complete result of this work will be published as soon as they finish this project.

The idea of how to deal with the people coming from many places in the world are the other experience that I got. I also had a great opportunity to know a few researchers in the other field rather than only the ones who are interested in carcinogenesis. They came from other places than USA, such as Japan, Taiwan, Korea and Europe. I hope to use this experience to strengthen my own lab in the very near future.

ACKNOWLEDGEMENT

I would like to give appreciated thanks to Thailand Research Fund and Ericsson Foundation, Thailand for their funding from the Thai's side. The other appreciated thanks will be given to the Asian Partnership Initiative, UW-Madison and McArdle Lab for Cancer Research for the funding from the USA's side. Prof. James A. Will is given a grateful thanks for his role in

making the collaboration between UW-Madison and Khon Kaen University and for his kindly introduction of me to my mentor.

I also would like to give the appreciated thank to Prof. Norman Drinkwater for allowing me to work on the postdoctoral project in his lab. Big thanks will be given to the other members in Drinkwater's lab i.e. Andrea Bilger, Mara Feld, Stephanie Peychal, Mei Finnerty, Jennifer Drew, Bret Williams, and Tonia Sundlov for their kindly help in the lab. Special thanks is given to Department of Biochemistry, Faculty of Medicine, Khon Kaen University for allowing me spend 1-year postdoctoral training at the University of Wisconsin-Madison, USA. Finally, I really would like to say thank you to my mother, my husband and my lovely son for giving me the influential encouragement.

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Estimates of worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993; 54: 549-606
2. Richardson HL, Griffin AC, Rinfret AP. Adrenal histological change and liver-tumor inhibition in hypophysectomized rats fed the azo dye, 3-methyl-4-dimethylaminoazobenzene. Cancer 1953; 6: 1025-1029
3. Griffin AC, Rinfret AP, Corsiglia VF. The inhibition of liver carcinogenesis with 3-methyl-4-dimethylaminoazobenzene in hypophysectomized rats. Cancer Res. 1954; 13: 77-79
4. Orian JM, Lee CS, Weiss LM, Brandon MR. The expression of a metallothionein-ovine growth hormone fusion gene in transgenic mice does not impair fertility but results in pnthological lesions in the liver. Endocrinology 1989; 124: 455-463
5. Orian JM, Tamakoshi K, Mackay IR, Brandon MR. New murine model for hepatocellular carcinoma: transgenic mice expressing metallothionein-ovine growth hormone fusion gene. J Natl Cancer Inst. 1990; 82: 393-398
6. Eicher EM, Beamer WG. Inherited ateliotic dwarfism in mice. Characteristics of the mutation, little, on chromosome 6. J Hered. 1976; 67: 87-91
7. Godfrey P, Rahal JQ, Beamer WG, Copeland NG, Jenkins NA, Mayo KE. GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor receptor function. Nature Genet. 1993; 4: 227-232
8. Lin SC, Lin CR, Gukovesky I, Lussi AJ, Sawchenko PE, Rosenfeld MG. Molecular basis of the little mouse phenotype and implications for cell type-specific growth. Nature 1993; 364: 208-213
9. Cheng TC, Beamer WG, Phillips JA. III, Bartke A, Mallonee RL, Dowling C. Eitiology of growth hormone deficiency in little, Ames and Snell dwarf mice. Endpcrinology 1983; 113: 1669-1678
10. Bugni JM, Drinkwater NR. The *little* mutation suppresses DEN-induced hepatocarcinogenesis in mice and abrolate genetic and hormonal modulation of susceptibility. Carcinogenesis 2001; 22: 1853-1862
11. Kouroumalis E, Skordilis P, Thermos K, Vasilaki A, Moschandrea J, Manousos ON. Treatment of hepatocellular carcinoma with octreotide: A randomized controlled study. GUT 1998; 42: 442-447
12. Weckbecker G, Briner U, Lewis I, Bruns C. SOM230: a new somatostatin peptidomimetic with potent inhibitory effects on the growth hormone/insulin-like growth factor-I axis in rats, primates, and dogs. Endocrinology 2002; 143: 4123-4130