日本財団補助金による
1999 年度日中医学協力事業報告書
－在留中国人研究者研究助成－

2000 年 3 月 14 日

財団法人 日中医学協会
理事長 中島 章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ 肝癌、胆道癌における細胞周期Ｇ１期制御因子及び癌抑制遺伝子の異常の解明

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

第５８回日本癌学会 肝外胆管癌におけるｐ２１(WAF1/CIP1) 蛋白の発現低下とｐ５３の異常

厚生省研究班班会議 肝外胆管癌におけるｐ２１とｐ５３の発現異常

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

1. 著者：Ai-Min Hui, Xin Li, Masatoshi Makuuchi, Tadatoshi Takayama and Keiichi Kubota
題名：Over-expression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma
雑誌：Int. J. Cancer: 84, 604-608 (1999)

2. 著者：Xin Li, Ai-Min Hui, Tadatoshi Takayama, Xing Cui and Masatoshi Makuuchi
題名：Altered $p21^{WAF1/CIP1}$ expression is associated with poor prognosis in extrahepatic bile duct carcinoma
雑誌：Cancer letter: in press

3. 著者：Xin Li, Ai-Min Hui, Ya-Zhou Shi, Tadatoshi Takayama and Masatoshi Makuuchi
題名：Reduced $p21^{WAF1/CIP1}$ expression participates in the early development stage of gallbladder carcinoma and associates with p53 overexpression
雑誌：投稿中

3．今後の研究計画

サイクリン依存性キナーゼインヒビター(CKI) は二つのファミリーに大別される。一つはｐ１６を代表とするｐ１６、ｐ１５、ｐ１８とｐ２０のファミリーで、もう一つはｐ２１を代表とするｐ２１、ｐ２７とｐ５１のファミリーである。ｐ１６ファミリー CKI の遺伝子は皆 CpG islands という結合を持っている。 CpG islands がメチル化されることによって、これらの因子の蛋白発現が低下し、細胞周期進行を負に調節機能が抑制されることになる。これから、我々は Methylation-specific PCR 法を用いて、肝発癌における細胞周期制御因子 p14,p15,p16,p19 のメチル化異常の役割に関する研究をする予定であります。

4．研究指導者の意見

李キン君は平成１０年４月より本学大学院医学博士課程に入学し、私の元で肝癌及び胆道癌の発癌メカニズムに関する研究を行っております。 肝外胆管癌における p21 と p53 の異常に関する論文 “ Altered p21 expression is associated with poor prognosis in extrahepatic bile duct carcinoma” が Cancer Letter 誌に採用され、本研究成果を第 58 回日本癌学会総会で発表しました。また、胆嚢癌に関する論文“ Reduced p21 expression participates in the early developmental stage of gallbladder carcinoma and associates with p53 overexpression” を英文誌に投稿中であります。さらに、第二著者としての論文が International Journal of Cancer 誌 (1999,84:604-608) に発表されました。

李君は非常に真面目で勤勉で生活態度も大変真面目で人柄は温厚で明るく協調性があり、教室員からも信頼されており、人間的にも優れた資質を有しております。李君は必ずや将来優れた研究者として、日本と中国の架け橋となることでしょう。

研究指導者氏名　幕内　雅敏　㊞

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）
タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。
研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。
論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

肝外胆管癌における $p21^{WAF1/CIP1}$ と p53 の発現異常

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要　旨

[目的] 肝外胆管癌における CDK インヒビター $p21^{WAF1/CIP1}$ の発現異常の臨床的意義及び p53 との関連について検討した。 [材料と方法] 外科的に切除された肝外胆管癌 34 例のホルマリン固定パラフィン包埋標本を対象とし、抗 $p21^{WAF1/CIP1}$ 抗体及び抗 p53 抗体を用い、免疫組織染色を行った。 $p21^{WAF1/CIP1}$ 染色では染色された癌細胞が 10%未満を低発現($p21^{WAF1/CIP1}$–)とし、10%から 30%未満を中等度発現($p21^{WAF1/CIP1}$+)とし、30%以上を高発現($p21^{WAF1/CIP1}$++) とした。 p53 染色では染色された癌細胞が 20%以上を異常発現とした。[結果] 肝外胆管癌 34 例中 23 例(67.6%)において $p21^{WAF1/CIP1}$ の低発現、6 例(17.6%)において中等度発現、5 例(14.8%)において高発現が認められた。 TNM stage との関係を見ると、$p21^{WAF1/CIP1}$ 中等度発現群と比べて、低発現群では stageIII,IV の症例が有意に高率でした(p=0.007)。 また、$p21^{WAF1/CIP1}$ 低発現群及び高発現群は中等度発現群より有意に予後不良であった($p21^{WAF1/CIP1}$– *versus* $p21^{WAF1/CIP1}$+, p=0.02; $p21^{WAF1/CIP1}$++ *versus* $p21^{WAF1/CIP1}$+, p=0.01)。 p53 の発現は肝外胆管癌 34 例中 17 例(50%)で異常であり、静脈浸潤との相関が見られた(p=0.001)。 $p21^{WAF1/CIP1}$ の発現と p53 との関連性が認められなかった。[結語] 1. 肝外胆管癌において $p21^{WAF1/CIP1}$ 発現低下及び発現過剰は予後不良に関与する。 2. $p21^{WAF1/CIP1}$ の発現が p53 非依存的である。

KEY WORDS： $p21^{WAF1/CIP1}$、 p53、 肝外胆管癌、予後。

RESEARCH REPORT

PURPOSE

Recently, a body of evidence has revealed a direct link between cell cycle control and tumorigenesis. The molecular machinery of the cell cycle consists of a series of positive regulators such as cyclins and cyclin-dependent kinases (CDKs), and a group of negative regulators known as CDK inhibitors (CKIs), which include $p21^{WAF1/CIP1}$, $p27^{Kip1}$ and $p16^{INK4}$. $p21^{WAF1/CIP1}$ binds to a broad range of cyclin–CDK complexes and blocks their catalytic activity. Moreover, there are several pieces of evidence to show that $p21^{WAF1/CIP1}$ protein suppresses tumor growth, suggesting that $p21^{WAF1/CIP1}$ may act as a tumor suppressor protein. Although it has been clarified that the $p21^{WAF1/CIP1}$ gene mutation is extremely rare in a variety of human malignancies, alteration in the expression of the $p21^{WAF1/CIP1}$ protein has been reported in a variety of human cancers. However, no studies have been published on the expression of $p21^{WAF1/CIP1}$ in EBDC so far. $p21^{WAF1/CIP1}$ expression was initially considered to be transcriptionally regulated by functional p53. The aim of this study was to determine the possible clinical role of $p21^{WAF1/CIP1}$ protein expression and whether $p21^{WAF1/CIP1}$ expression correlates with p53 status in EBDC.

METHODS

Patients and specimens Tumor specimens were obtained from 34 patients with EBDC who underwent surgical resection at our university from October 1990 to March 1998. The median age was 65 years (range 43–88 y). There were 2 stage I, 6 stage II, 4 stage III, and 22 stage IVA tumors (TNM stage).

Immunohistochemistry Immunostaining for $p21^{WAF1/CIP1}$ and p53 was done on archival, formalin-fixed, paraffin -embedded tissue sections using the avidin–biotin–peroxidase method. Antigens were retrieved with 10mM citrate buffer (pH 6.0) by an autoclave method. Either

anti-$p21^{WAF1/CIP1}$ monoclonal antibody EA10 (Oncogene Science, Cambridge, MA) or anti-p53 monoclonal antibody DO-7 (Dako, A/S, Denmark), diluted 1:100, was applied to the sections. Then the sections were incubated with biotinylated secondary antibody and then with avidin-biotinylated horseradish peroxidase complex. Peroxidase staining was done using 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. Only when unequivocal nuclear staining was observed, cells were considered positive staining. Three categories were used to describe the extent of $p21^{WAF1/CIP1}$ immunoreactivity: –, negative expression (low-level expression, <10% positive carcinoma cells); +, positive expression (moderate expression, 10%–30% positive carcinoma cells); ++, strongly positive expression (high-level expression, ≥30% positive carcinoma cells). For p53, tumors with ≥20% p53 immunoreactive cells were defined as p53-positive (p53 aberrant pattern); tumors with <20% p53 immunoreactive cells were defined as p53-negative (p53 wild-type pattern).

Statistical analysis The χ^2 test was used to analyze the possible correlation between $p21^{WAF1/CIP1}$ and p53 expression and the associations of $p21^{WAF1/CIP1}$ and p53 expression with clinicopathologic parameters. Survival curves were calculated using the Kaplan–Meier method, and the statistical significance of the differences between the curves was evaluated by the log rank test. A p value < 0.05 was considered statistically significant.

RESULTS

$p21^{WAF1/CIP1}$ expression Of the 34 EBDCs, 23 (68%) showed low-level $p21^{WAF1/CIP1}$ expression ($p21^{WAF1/CIP1}$–), 6 (18%) showed moderate $p21^{WAF1/CIP1}$ expression ($p21^{WAF1/CIP1}$+), and the remaining 5 (15%) showed high $p21^{WAF1/CIP1}$ expression ($p21^{WAF1/CIP1}$++). According to the Kaplan–Meier survival analysis, patients with $p21^{WAF1/CIP1}$– and $p21^{WAF1/CIP1}$++ tumors had significantly shorter disease-free survival than patients with $p21^{WAF1/CIP1}$+ tumors ($p21^{WAF1/CIP1}$– *versus* $p21^{WAF1/CIP1}$+, $p = 0.02$; $p21^{WAF1/CIP1}$++ *versus* $p21^{WAF1/CIP1}$+, $p = 0.01$; log rank test). No significant difference between $p21^{WAF1/CIP1}$– and $p21^{WAF1/CIP1}$++ tumors was detected in disease prognosis.

$p21^{WAF1/CIP1}$– was significantly associated with advanced disease stage (occurring in 25% of stage I/II tumors, 75% of stage III, and 82% of stage IVA), while $p21^{WAF1/CIP1}$+ was more common in lower-stage tumors (occuring in 50% of stage I/II tumors, 0% of stage III, and 9% of stage IVA; $p21^{WAF1/CIP1}$– *versus* $p21^{WAF1/CIP1}$+, $p = 0.007$). No significant relationship was detected between $p21^{WAF1/CIP1}$ expression level and the other clinicopathologic parameters assessed, including histologic type, venous invasion, and tumor site.

p53 expression Half of the 34 EBDCs showed p53-positive expression. p53-positive expression was significantly related to venous invasion ($p = 0.001$). No significant difference in survival was found between patients with p53-positive tumors and those with p53-negative tumors ($p = 0.17$, log rank test).

Relationship between $p21^{WAF1/CIP1}$ and p53 expression Seventy-one percent of p53-positive and 65% of p53-negative tumors showed $p21^{WAF1/CIP1}$-negative expression, while 35% of p53-negative and 29% of p53-positive tumors showed $p21^{WAF1/CIP1}$-positive expression. These data indicate no significant correlation between $p21^{WAF1/CIP1}$ and p53 expression ($p = 0.71$).

DISCUSSION

We have shown that not only reduced expression but also overexpression of $p21^{WAF1/CIP1}$ protein exerted adverse influence on the clinical outcome of EBDC. By contrast, tumors showing moderate $p21^{WAF1/CIP1}$ expression were significantly related to increased patient survival. It was reported that reduced $p21^{WAF1/CIP1}$ expression correlated with poor prognosis in gastric and other cancers. On the other hand, in esophageal cancer and so on, $p21^{WAF1/CIP1}$ overexpression was considered to relate to unfavorable outcome. Consistent with our results, a similar finding was reported recently for bladder cancer.

$p21^{WAF1/CIP1}$ acts as a tumor growth suppressor. So the great proportion of EBDCs (23/34 tumors) showed low $p21^{WAF1/CIP1}$ expression was significantly associated with advanced stage as well as with increased recurring disease, confirming abnormal downregulation of $p21^{WAF1/CIP1}$ expression play a role in disease progression.

Unexpectedly, the finding that patients with tumors with elevated $p21^{WAF1/CIP1}$ expression had the worst prognosis indicated that overexpressed $p21^{WAF1/CIP1}$ protein does lose its tumor-suppressing functions. These findings suggest that there are multiple mechanisms involved in $p21^{WAF1/CIP1}$-related tumorigenesis in EBDC.

The mechanisms that allow tumor progression in the presence of high $p21^{WAF1/CIP1}$ expression remain to be determined. The first possibility is inactivation of the inhibitory function of $p21^{WAF1/CIP1}$. It has been reported that a viral oncoprotein, HPV-16 F7, can block the ability of cell growth arrest of $p21^{WAF1/CIP1}$, inducing abnormal cell growth despite high level of $p21^{WAF1/CIP1}$. In the second possibility, it is important to point out that cell cycle control is a complex balance between the level of positive and negative cell cycle regulators. High $p21^{WAF1/CIP1}$ expression may be accompanied by an even higher level of positive cell cycle regulators, which may be the real cause of the tumor progression. In this case, although $p21^{WAF1/CIP1}$ expression is upregulated to a high degree, CKIs are still not sufficient to overcome the activities of positive regulators.

In liver and other cancers, $p21^{WAF1/CIP1}$ expression was considered to be regulated through a p53-dependent pathway. However, we failed to find a significant association between $p21^{WAF1/CIP1}$ and p53 expression in EBDC. This suggests that, even if p53 participates in the regulation of $p21^{WAF1/CIP1}$ expression for a proportion of EBDCs, there should be p53-independent mechanisms involved in the process of $p21^{WAF1/CIP1}$ regulation. Many recent studies have clarified that $p21^{WAF1/CIP1}$ expression is also regulated by a p53-independent pathway.

EBDC is known to have poor prognosis and to be difficult to cure. To date, little is understood about the molecular basis of tumorigenesis for this cancer type. This study indicates that reduced expression or overexpression of $p21^{WAF1/CIP1}$ plays an adverse role in tumor progression and clinical outcome for this disease. Evaluation of $p21^{WAF1/CIP1}$ level may be important in determining the biological behaviors of EBDC, and may provide useful information in managing patients with this devastating illness.

REFERENCES

El-deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J.M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, 75, 817-825 (1993).

Miller, F. R. Tumor suppression by $p21^{WAF1}$. Cancer Res., 55, 4536-4539 (1995).

Shiohara, M., El-deiry, W. S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D. L., Vogelstein, B. and Koeffler, H. P. Absence of WAF1 mutations in a variety of human malignancies. Blood, 84, 3781-3784 (1994).

Gomyo, Y., Ikeda, M., Osaki. M., Tatebe, S., Tsujitani, S., Ikeguchi, M., Kaibara, N. and Ito, H. Expression of p21 (waf1/cip1/sdi1), but not p53 protein, is a factor in the survival of patients with advanced gastric carcinoma. Cancer, 79, 2067-2072 (1997).

Stein, J. P., Ginsberg, D. A., Grossfeld, G. D., Chatterjee, S. J., Esrig, D., Dickinson, M. G., Groshen, S., Taylor, C. R., Jones, P. A., Skinner, D. G. and Cote, R. J. Effect of $p21^{WAF1/CIP1}$ expression on tumor progression in bladder cancer. J. Natl. Cancer Inst., 90, 1072-1079 (1998).

Diamantis, I., Karamitopoulou, E., Perentes, E. and Zimmermann, A. p53 protein immunoreactivity in extrahepatic bile duct and gallbladder cancer: correlation with tumor grade and survival. Hepatology, 22, 774-779 (1995).

Suto, T., Sugai, T., Nakamura, S., Funato, O., Nitta, H., Sasaki, R., Kanno, S. and Saito, K. Assessment of the expression of p53, MIB-1 (Ki-67 antigen), and argyrophilic nucleolar organizer regions in carcinoma of the extrahepatic bile duct. Cancer, 82, 86-95 (1998).

Japanese Society of Biliary Surgery "General rules for surgical and pathological studies on cancer of biliary tract," 3th ed. (1993). Kanehara & Co., Ltd., Tokyo.

Sobin L. H. and Wittekind C. "TNM classification of malignant tumors, " 5th ed. (1997). Wiley-Liss, Inc., New York.

Sarbia, M., Stahl, M., Hausen, A. Z., Zimmermann, K., Wang, L., Fink, U., Heep, H., Dutkowski, P., Willers, R., Muller, W., Seeber, S. and Gabbert, H. E. Expression of $p21^{WAF1}$ predicts outcome of esophageal cancer patients treated by surgery alone or by combined therapy

modalities. Clin. Cancer Res., 4, 2615-2623 (1998).

Funk, J. O., Waga, S., Harry, J. B., Espling, E., Stillman, B. and Galloway, D. A. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev., 11, 2090-2100 (1997).

Hui, A.-M., Kanai, Y., Sakamoto, M., Tsuda, H. and Hirohashi, S. Reduced $p21^{WAF1/CIP1}$ expression and p53 mutation in hepatocellular carcinomas. Hepatology, 25, 575-579 (1997).

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. and Wang, X. F. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. USA, 92, 5545-5549 (1995).