17

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※

※　　　　　　　　　　　　　　　　　　　　　　　　　※

※　p73 和 p51 在腫瘤發生及動物發育中扮演的角色　※

※　Roles of p73 and p51 in tumorigenesis and animal development　※

※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：□個別型計畫　　□整合型計畫

計畫編號：NSC89－2320－B－040－018－

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

計畫主持人：潘惠錦

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：私立中山醫學院

中　華　民　國　八十九年　十月　二十七日

行政院國家科學委員會專題研究計劃成果報告

p73 和 p51 在腫瘤發生及動物發育中扮演的角色
Roles of p73 and p51 in tumorigenesis and animal development

計劃編號：NSC 89-2320-B-040-018
執行期限：88 年 8 月 1 日至 89 年 7 月 31 日
主持人：潘惠錦　中山醫學院 生命科學系
Email: hp29@mercury.csmc.edu.tw

一、中英文摘要

在過半的人類癌症中皆發現有抑癌基因 p53 的突變。近來發現一個新的 p53 相似物，稱為 p73，它具有與 p53 的 DNA 結合、轉活化及寡聚物聚合部位非常高度的序列相似性。研究顯示 p73 具有與 p53 相似的寡聚合及轉活化能力，並且 p73 可活化 p53-感應基因如 p21，並可經由引發細胞凋亡來抑制細胞生長。為探討 p73 是否與肝細胞癌的病因有關，我們檢查了 18 對正常及肝癌組織在 p73 基因的基因座缺失、基因座不平衡表達、以及基因突變等情況。PCR-RFLP 分析其 exon 2 的多形性顯示，在 15 個正常檢體中有 5 個是異形合子，而其中沒有任何一個在相對的癌組織中有缺失。檢查 RNA 的樣本顯示兩個基因座的 RNA 皆存在於 5 個異形合子，顯示 p73 基因在肝組織是雙基因座表達。有趣的是，p73 基因表達的量在癌組織中總是比在相對的正常組織中高。最後，單股構形多形性及定序分析顯示在外顯子 6、9、14 有數個多形性及在內顯子 6、9 也有一些變異。但是在整個 p73 的編碼區域並沒有任何的突變。這些結果指出 p73 並不像 p53 一樣在癌腫中扮演抑癌的角色，反而大量的表達 p73 基因可能與肝癌的病因有關。

關鍵詞：p73、基因座表達、突變、肝癌

Abstract

Mutations in the p53 tumor suppressor gene have been found in about half of all human cancers. Recently, a novel gene encoding a protein, termed p73, was identified with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. It was shown that p73 possess oligomerization and transactivation properties similar to p53 and it could activate p53-responsive genes such as p21, and suppress cell growth by inducing apoptosis. To investigate whether p73 is involved in the pathogenesis of hepatocellular carcinoma, we examined the presence of allelic loss, allelic expression imbalance and mutations of p73 gene in 18 pairs of normal and hepatocellular cancer tissues. PCR-RFLP analysis of a polymorphism in exon 2 revealed that 5 out of 15 normal samples were heterozygous and none of them were lost in the cancer counterparts. Both alleles were present in RNA samples from the five heterozygous individuals, indicating that p73 was biallelically expressed in the liver tissue. Interestingly, the expression level of p73 was consistently high in the cancerous tissues, in contrast to very low expression in the paired normal tissues. Finally, single strand conformation polymorphism and sequencing analysis revealed several polymorphisms in exon 6, 9, 14, as well as some variations in intron 6 and 9, but no mutations were found in the coding sequence of the p73 gene. These results indicate that p73 does not play a role in suppressing tumor growth as p53 does, but overexpression of p73 may somehow contribute to the pathogenesis of hepatocellular carcinoma.

Keywords: p73, allelic expression, mutation hepatocellular carcinoma

二、緣由與目的

Mutations in the p53 tumor suppressor gene have been found in about half of all human cancers. The possible mechanisms by which p53 inactivation contributes to tumorigenesis have been linked to its growth suppressive and apoptosis-inducing activity. Loss of p53 function appears to confer selective advantages on cell growth through deregulated growth and resistance to cell death.

For nearly two decades, p53 has been regarded as an orphan with no family proteins or relatives. But recently, several novel genes encoding proteins with remarkable sequence homology to p53 and functionally resembles p53 were cloned (Kaghad et al., 1997; Osada et al., 1998; Trink et al. 1998; Yang et al., 1998; Senoo et al., 1998). One of these genes was termed p73. The homology between p73 and p53 is extensive within the most conserved p53 domains involved in transactivation, DNA binding, and oligomerization (Kaghad et al, 1997). Significantly, residues that correspond to the mutational hot spots in tumors are conserved in p73. It was shown that p73 has oligomerization and transactivation activities similar to p53. Moreover, p73 could activate p53-responsive genes such as p21, inhibit cell growth and induce apoptosis in a p53-like manner in cultured cells (Jost et al, 1997).

p73 was mapped to chromosome 1p36, a region which is frequently deleted in neuroblastoma and other tumors. Based on this finding and the striking similarities of p73 with p53, it is tempting to postulate that p73 may act as a tumor suppressor like p53 does. However, recent studies have shown that allelic loss of 73 was rare and mutations in the p73 gene were not found in a variety of human cancers. In some cases, activation of the silent allele or overexpression of the wild type p73 was even reported. These data suggested a totally contrary role of p73 as a tumor suppressor.

Originally we proposed a three-year study to investigate the potential involvement of p73 and p51 in the pathogenesis of human cancers, and to investigate the biological function of p73 and p51 in cell differentiation and development. However, the reviewer did not favor the proposed aim two and thus the grant is cut into a one-year study. We therefore performed most works as stated in specific aim one but not aim two. Because loss of heterozygosity on chromosome 1p36 was found in 30%-33% of hepatocellular carcinomas (Yeh et al. 1994; Kuroki et al. 1995), we investigated the role of p73 alteration in the pathogenesis of hepatocellular carcinoma by examining the presence of allelic loss, allelic expression imbalance and mutations of the p73 gene in hepatocellular carcinomas.

三、結果與討論

We have collected 18 pairs of matched normal and hepatocellular cancer tissues, isolated their genomic DNAs and RNAs. To determine if there is loss of heterozygosity in the p73 gene, we first PCR-amplified a genomic fragment containing part of exon 2 and then checked a polymorphism in exon 2 by restriction fragment length polymorphism (RFLP) analysis. The result revealed that 5 out of 15 normal samples were heterozygous and none of them were lost in the cancer counterparts (Pan et al. manuscript in preparation; table 1 and fig.1).

Further analysis of the RNA samples from matched normal and cancer tissues by RT-PCR-RFLP assays revealed that both alleles were present in RNA samples from the five heterozygous individuals, indicating that

p73 was biallelically expressed in the liver tissue (table. 1). This is different from what was reported in the peripheral blood, where monoallelic expression of 73 was observed. Thus, monoallelic expression could not possibly account for the mechanism of LOH, at least in hepatocellular carcinomas.

Since there was no LOH and the gene was biallelically expressed, we then performed single strand conformation polymorphism (SSCP) and sequencing analysis to look if there is any mutations present. We synthesized 7 pairs of exon primers which amplified 7 overlapping fragments encompassing the mRNA, and 14 pairs of intron primers which amplified the 14 exons. Products from either RT-PCR or genomic PCR were analyzed by SSCP and the altered bands were then sequenced. Summation of the results revealed several polymorphisms in exon 6, 9, 14, as well as some variations in intron 6 and 9, but no mutations were found in the entire coding sequence of the p73 gene (table 2).

Most interestingly, however, we have observed that the expression level of p73 was consistently high in the cancerous tissues, in contrast to very low expression in the paired normal tissues (fig. 2). The elevated expression of p73 in tumor over normal tissues has also been reported in breast (Zaika et al. 1999), bladder (Chi et al. 1999; Yokomizo et al. 1999), and brain tumors (Loiseau et al. 1999). In addition, activation of a silent allele and overexpression of wild type p73 has been observed in some lung cancers (Mai et al. 1998). Whether overexpression of p73 contributes to the pathogenesis or progression of these tumors awaits further investigation. Our data, together with those from others, indicate that p73 may hardly function as a tumor suppressor in a classic Knudson's manner. On the contrary, overexpression of p73 may somehow contribute to the pathogenesis of hepatocellular carcinomas.

四、成果自評

The research described above basically follows the outlines presented in the original project except that studies related to p51 were deleted. This is largely due to the limitation in manpower as well as the consideration of its compatibility in this field. We have, however, continued to work on the expression of p73. Based on the findings from this project, we are now expanding our efforts on exlporing the mechanisms regulating p73 gene expression, including methylation and promoter/enhancer studies. We are also looking for the non-mammalian homolog of p73 and trying to uncover its role in development.

五、參考文獻

1. Chi SG, et al. 1999. Elevated and biallelic expression of p73 is associated with progression of human bladder cancer. *Cancer Res.* 59, 2791-2793.
2. Jost, A.C., Marin, C. M. and Kaelin Jr., G.W. 1997. p73 is a human p53-related protein that can induce apoptosis. *Nature* 389,191-194.
3. Kaghad, M. et al. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 90, 809-819.
4. Kuroki, T. et al. 1995. Accumulation of genetic changes during development and progression of hepatocellular carcinoma: loss of heterozygosity on chromosome arm 1p occurs at an early stage of hepatocarcinogenesis. *Genes, Chrom. Cancer* 13, 163-167.
5. Loiseau H, et al. 1999. p73 gene transcripts in human brain tumors: overexpression and altered splicing in ependymomas. *Neurosci Lett.* 263, 173-176.
6. Mai, M. et al. 1998a. Activation of p73 silent allele in lung cancer. *Cancer Res.* 58, 2347-2349.
7. Osada M. et al. 1998. Cloning and

functional analysis of human p51, which structurally and functionally resembles p53. *Nature Med.* 4, 839-843.

8. Senoo, M. et al. 1998. A second p53-related protein, p73L, with high homology to p73. *Biochem. Biophys. Res. Com.* 248, 603-607.
9. Trink, B. et al. 1998. A new human p53 homologue. *Nature Med.* 4, 747-748.
10. Yang, A. N. et al. 1998. P63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Molecular cell* 2, 305-316.
11. Yeh, S-H. et al. 1994. Frequent genetic alterations at the distal region of chromosome 1p in human hepatocellular carcinomas. *Cancer Res.* 54, 4188-4192.
12. Yokomizo A, et al. 1999. Overexpression of the wild type p73 gene in human bladder cancer. *Oncogene.* 18, 1629-1633.
13. Zaika AI, et al. 1999. Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. *Cancer Res.* 59:3257-3263.

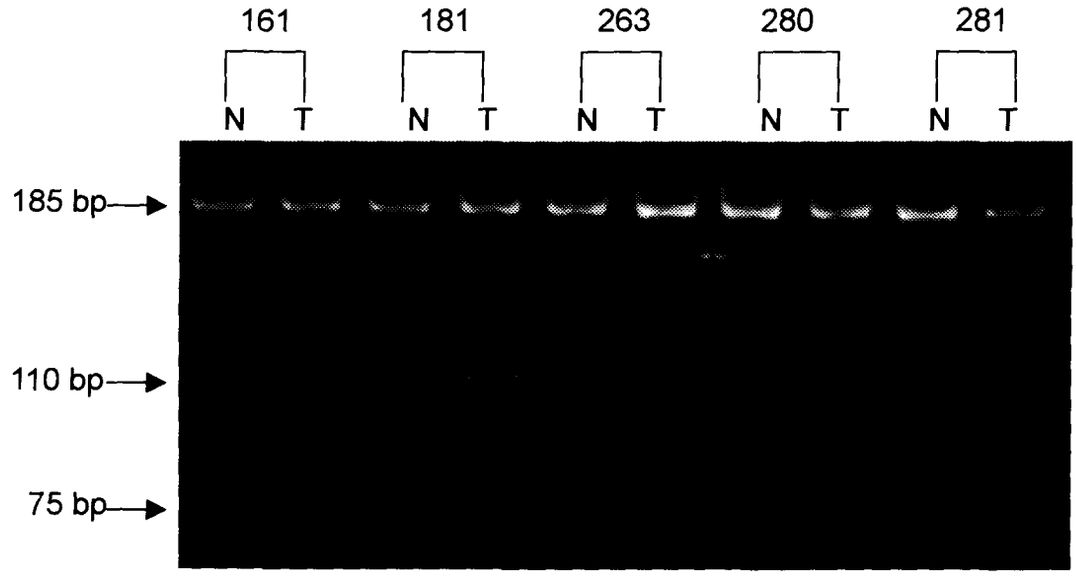

Fig. 1 Examination of the allelic loss at the p73 locus in representative HCCs. Genomic DNA was PCR amplified and yielded a product of 185 bp which contained a GC/AT polymorphism. Sty I digestion of PCR product derived from the AT allele resulted in two smaller fragments of 110 bp and 75 bp, whereas PCR product derived from the GC allele remained uncut. Lanes 1, 2, 3, 4, 7, 8 indicate heterozygous GC/AT alleles and lanes 5, 6, 9, 10 indicate homozygous GC/GC alleles. N: normal; T: tumor. Note that the three heterozygous patients (161, 181, 280) did not show allelic loss at the p73 locus.

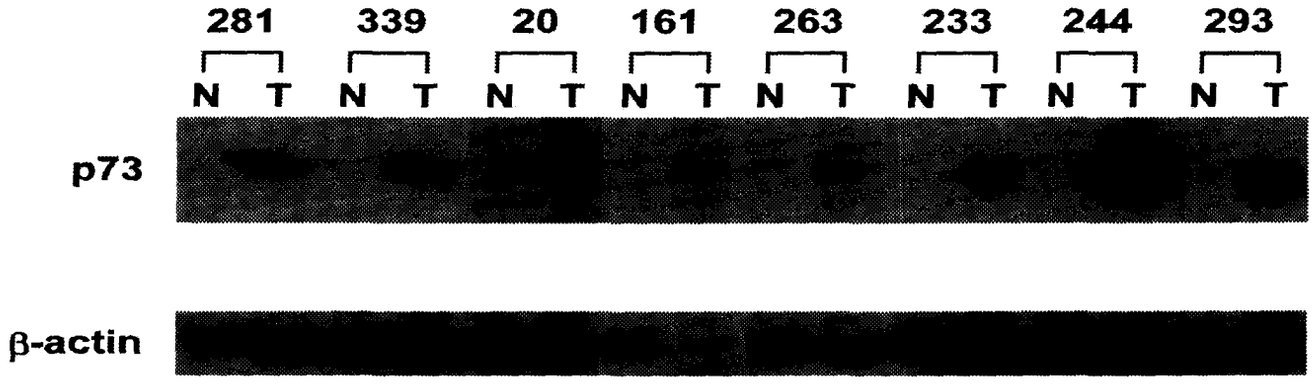

Fig. 2 Expression of p73 by RT-PCR analysis in representative HCCs of paired normal (N) and tumor (T) tissues. Note that the expression levels of p73 vary among patients but in each case the expression is high in tumor tissue as compared to the negligible expression in normal tissue.

Table 1 Alleleotyping and allelic expression of p73 in normal and hepatocellular carcinoma tissues

Patient	DNA		RNA	
Number	Normal	Tumor	Normal	Tumor
20	GC/GC	GC/GC		
115	GC/GC	GC/GC		
126	GC/AT	GC/AT	GC/AT	GC/AT
127	GC/GC	GC/GC		
149	GC/GC	GC/GC		
161	GC/AT	GC/AT	GC/AT	GC/AT
181	GC/AT	GC/AT	GC/AT	GC/AT
216	ND	ND	GC/AT	GC/AT
233	ND	ND	GC/AT	ND
244	ND	ND	GC/GC	ND
250	ND	ND	GC/AT	ND
263	GC/GC	GC/GC		
280	GC/AT	GC/AT	GC/AT	GC/AT
281	GC/GC	GC/GC		
293	ND	ND	GC/GC	ND
333	GC/GC	GC/GC		
339	GC/GC	GC/GC		
401	GC/AT	GC/AT	GC/AT	GC/AT

ND:not determined

Table 2 Polymorphism of p73 gene identified in HCC

Nucleotide number	Exon/Intron	Nucleotide change	Amino Acid
nt 836	Exon 6	A→G	Pro→Pro
Exon 6+52	Intron 6	C→G	—
nt 1157	Exon 9	T→C	His→His
Exon 9+24	Intron 9	A→G	—
nt 1781	Exon 14	G→A	Ala→Ala
nt 1940	Exon 14	G→A	Ala→Ala