行政院國家科學委員會補助專題研究計畫成果報告

p73 基因的表達研究(2/2)

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC90-2320-B-040-034

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計畫主持人：潘惠錦

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行政院國家科學委員會專題研究計劃成果報告

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Expression study of the p73 gene(2/2)

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一、中文摘要

p73 是一個 p53 同源物，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，並經由引發細胞凋亡來抑制細胞生長。在之前的研究中，我們發現 p73 基因在肝細胞癌中並無突變，而且 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。在本研究中，我們著重探討 p73 基因的表達調控機制，包括甲基化及驅動子等之分析。首先我們分析在癌組織和正常組織中 p73 基因在驅動子及第一外顯子的甲基化模式。結果顯示，不管在癌組織或正常組織 p73 基因都沒有甲基化。其次，我們選殖了一段包含 p73 基因驅動子區域的 1.3 kb DNA 片段，並建構一系列連接 GFP 或 luciferase 報導基因的刪除序列。轉殖分析的結果顯示，1.3 kb 的片段含有最高的驅動子活性，但是 136 bp 的片段即已含有基本的活性。另外，我們也從斑馬魚的卵巢 RNA 選殖出 p73 cDNA。此 cDNA 包含一個可轉譯出 640 個胺基酸的 ORF (1923 bp)，它與鯰魚(Barbul barbus)、老鼠(Mus musculus)、人(Homo sapiens)、 猿猴(Cercopithecus aethiops)及蝸牛(Mya arenaria)的 p73 相似度分別為 95%、71% 、71%、70%、 32%。由 RT-PCR 結果得知，p73 的表現局限在皮膚、魚鰭、腦、卵巢、睪丸，與 p53 的廣泛表現顯著不同。在胚胎發育過程中 p73 RNA 在受精 3 小時後即可偵測到表現，直到 120 小時。利用全體原位雜交及免疫化學染色方法，發現 p73 也在頭部的嗅球及卵巢和睪丸的特定細胞上有表現。斑馬魚 p63 基因也已以同樣的方式選殖出。這些結果顯示 p73 可能參與卵巢和睪丸的某些特定功能，也可能與感覺器官的訊息傳遞有關。未來進一步實驗我們將使用 morpholino antisense oligo 來探討 p73 在斑馬魚胚胎發育時所扮演的角色。

關鍵詞：p73、甲基化、基因表達、斑馬魚發育

Abstract

p73, the p53 homologue, was shown to have remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. It possess oligomerization and transactivation properties similar to p53 and can activate p53-responsive genes such as p21, and suppress cell growth by inducing apoptosis. In the previous study, we found that p73 was not mutated in hepatocellular carcinomas and that the levels of p73 were elevated in the cancerous tissues as compared to their normal counterparts. In this study, we have worked on exploring the mechanisms regulating p73 gene expression, including methylation and promoter/enhancer studies. First, we analyzed the methylation

patterns in the promoter and exon 1 regions of p73 gene in cancerous and normal tissues. Results indicate that there are no methylations associated with either cancerous or normal samples. Secondly, we cloned a 1.3 kb DNA fragment of the p73 promoter region and made a series of deletion constructs fused to either GFP or luciferase reporter gene. Transient transfection analysis revealed that the 1.3 kb fragment had the highest promoter activity compared to the other shorter constructs but p-136 (-136 to exon 1) already had the basal promoter activity. Finally, we cloned the p73 cDNA from zebrafish ovary RNA. The consensus open reading frame (1923 bp) encodes a polypeptide of 640 amino acids which shares 95, 71, 71, 70 and 32% identity to the p73 of barbel, mouse, human, *Cercopithecus aethiops* (African green monkey) and *Mya arenaria*(sftshell), respectively. RT-PCR analysis revealed that zebrafish p73 was expressed in restricted tissues such as skin, fin, brain, ovary and testis, in contrast to the more ubiquitous expression of zebrafish p53. During embryonic development, expression of p73 was detected at 3 hours post fertilization (hpf), and thereafter through 120 hpf. By wholemount in situ hybridization and immunohistochemical staining, p73 was found expressed in the olfactory bulb –like region and in the granulosa/theca cell layer of the ovary and in the Leydig/Sertoli cells of the testis. Zebrafish p63 homologue has been cloned using the same strategy and expression profile also examined. These results indicated that p73 may participate in certain aspect of ovary/testis function, as well as in the signaling in the sensory organ. Further experiment using morpholino antisense oligo of p73 will be conducted to dissect the role of p73 in zebrafish development.

Keywords: p73, methylation, gene expression, zebrafish development

二、緣由與目的

p73 is a novel p53 homologue which is found to possess remarkable sequence similarities to the DNA-binding, transactivation, and oligomerization domains of p53 (1). This gene has been characterized extensively for its biological functions *in vitro*. It was shown that p73 possessed oligomerization and transactivation properties similar to p53. It can activate p53-responsive genes such as p21, and suppress cell growth by inducing apoptosis (2). However, p73 is not activated by radiation-induced DNA damage (1), and viral oncoproteins such as SV 40 Tag and adenovirus E1B do not physically interact with p73 (3). p73 has an additional conserved domain at its C-terminus which may have regulatory function. Moreover, oncoprotein MDM2 can bind p73 without targeted degradation (4). All these data suggest that there are functional differences between p73 and p53.

p73 is mapped to chromosome 1p36.33, a region which is found to be frequently deleted in neuroblastoma and other tumors (1). Whether alterations of p73 contribute to the development of human cancers have been tested by screening a large numbers of different cancers for mutations in the p73 gene. So far, extensive search has led to the conclusion that somatic mutations of the p73 gene are infrequent in various human cancers (5). Thus, p73 does not seem to play a role as a tumor suppressor in the development of human cancers. On the contrary, we and others have found that the levels of p73 are elevated in the tumor tissues as compared to their normal counterparts (6). This observation raises interesting questions as to how expression of p73 is regulated in normal versus tumor cells and whether elevated expression of p73 contributes to the progression of some tumors.

In contrast to the increased expression in some solid tumors, however, it has been shown that expression of p73 was negligible in a sizable proportion of lymphomas and

leukemias. Reduction of expression was correlated to hypermethylation in the 5' region of p73 (7,8), suggesting an important role of DNA methylation in transcriptional regulation. Tissue-specific methylation has been shown to correlate with tissue-specific gene expression (9). p73 was initially thought to be monoallelically expressed, a phenomena commonly linked with imprinting. But later it was found both monoallelically and biallelically expressed in a variety of normal/cancer tissues, indicating a complex tissue-specific regulation mechanism.

To explore the molecular mechanisms by which expression of p73 gene is regulated, we proposed to 1) look for the methylation pattern in the 5' region of p73 and determine if there is any aberrant or tissue-specific methylation associated with altered expression. 2) look for and analyze the tissue-specific regulatory sequences in the p73 promoter. 3) determine the expression profiles of p73 during vertebrate development.

三、結果與討論

1) Methylation analysis of the p73 gene. p73 was shown to be hypermethylated in exon 1 region in some lymphomas and leukemias, and this was correlated with its low or absence in expression in these malignancies. We therefore analyzed the methylation patterns of p73 in both exon 1 and the 5' promoter region to see if there is any methylation which may be correlated with p73's expression in hepatocellular carcinoma. DNAs isolated from normal and cancerous tissues were first digested with either methylation-sensitive enzyme HpaII or methylation-resistant isoschizomer MspI. The digested DNAs were then amplified by PCR using 2 primer sets: m1: 5'-GAAGGGGACGCAGCGAAAC-3', m2: 5'-CTACCTGCAGCCGTCGCAG-3', and m3: 5'-GACTTGGACGCGGCCAGCTG-3', m4: 5'-GTTTCGCTGCGTCCCCTTCG-3', which amplifies the potential CpG islands in exon 1 and the upstream promoter region, respectively. The result indicated that none of the normal and cancerous liver tissues were methylated in the two regions assayed. In contrast, DNAs from Raji and U937 cell lines, in which p73 has been shown to be methylated in the exon 1 region, were fully methylated in both regions (6). Thus, methylation does not seem to play a role in p73 expression at least in liver cells. This further supports the notion that there are complex tissue-specific regulation mechanisms that control the expression of p73.

2) Promoter analysis of the p73 gene. We have PCR amplified and cloned a DNA fragment of about 1.3kb which consist of the 5' promoter region and exon 1 (5'-UTR) of p73 gene. We then made a series of deletion constructs containing different lengths of p73 promoter fused to either green fluorescent protein (GFP) or the luciferase reporter gene. Transient transfection analyses were performed in HepG2 cells (a human hepatoma cell line) using lipofectamine reagent. A pCDNA-LacZ construct was cotransfected with p73 constructs to monitor and normalize the efficiencies of transfection. Preliminary results showed that p-1200 (-1200 to exon 1) had the highest promoter activity compared to the other constructs but p-136 (-136 to exon 1) already had the basal promoter activity. Since there are numerous transcription factor binding sites in this region, dissecting of these binding sites are necessary to understand the regulation mechanism. Further experiments will involve site-directed mutagenesis to test the roles of these putative cis-elements. In addition, cells other than HepG2 will also be used to test the tissue specificity.

3) Expression profiles of p73 during vertebrate development. The zebrafish is a very good model for studying vertebrate development. We have therefore set up the culturing and breeding system of zebrafish in the lab. RNAs were isolated from different

tissues and RT-PCR analysis performed using primers designed from the conserved sequence in p73 of other species. The result indicated that p73 homolog was most abundant in the ovary. From RT-PCR and 3'-RACE, we amplified 4 overlapping fragments of about 570 bp, 1200 bp, 220 bp and 640 bp. (Fig. 1) Sequencing and analysis of these fragments revealed a consensus sequence of 2398 bp that contains an open reading frame (ORF) of 1923 bp and 37 bp and 437 bp in 5' and 3'-UTR region, respectively. This ORF encodes a polypeptide of 640 aa which shares 95, 71, 71, 70 and 32% identity to the p73 of barbel, mouse, human, *Cercopithecus aethiops* (African green monkey) and *Mya arenaria* (sftshell), respectively (Table I). To look for the expression profile of p73, RNA was isolated from different adult tissues and RT-PCR analysis was carried out. The result showed that zebrafish p73 was expressed in skin, fin, brain, ovary and testis. Lower expression was also detected in spleen and liver but not in other tissues. In contrast to this restricted expression pattern, zebrafish p53 was detected in most tissues examined (Fig. 2). During embryonic development, p53 was detected as early as 2 hours post fertilization (hpf), suggesting that it is a maternal effect gene. However, expression of p73 was not detected until 3 hpf, but was on thereafter through 120 hpf (Fig 3). By wholemount in situ hybridization and immunohistochemical staining, p73 was found expressed in the olfactory bulb –like region and in the granulosa/theca cell layer of the ovary and in the Leydig/Sertoli cells of the testis (Fig 4). This expression pattern of p73 in ovary/testis was also conserved in the mouse, suggesting a functional role of p73 in these organs. Zebrafish p63 homologue has been cloned using the same strategy and expression profile also examined.

四、成果自評

The research described above basically follows the outlines presented in the original project and is in the proposed timeframe. We have finished the experiments proposed in specific aim 1, and results are being published (6). As to specific aim 2, due to lack of manpower and inconsistency in the transfection efficiency, the promoter study has been delayed. We have directed our attention to specific aim 3 but are planning to use a different system to study the promoter activity when the manpower is not limited. So far, no one has published the sequence of p63 and p73 in zebrafish. We have cloned the zebrafish p73 and p63, studied the expression profile during development and in adult tissues. The next will be addressing their biological roles in development directly by disruption of their function. This will be done by injecting the morpholino antisense oligos into fish embryos and following the courses of development. Manuscript of this work is under preparation.

五、參考文獻

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Table I.

Table 1. Comparison of zebrafish p73 amino acid sequence with that of different species.

	Homo sapiens p73 alpha	Cercopithecus aethiops p73 alpha	Mus musculus p73 alpha	Barbul Barbus p73	Mya arenaria p73
Zebrafish p73: 640 aa	71%	70%	71%	95%	32%
Zebrafish p73: 139-318 aa*	90%	90%	90%	97%	57%

*Amino acids 139-318 represents the 'putative' DNA-binding domain

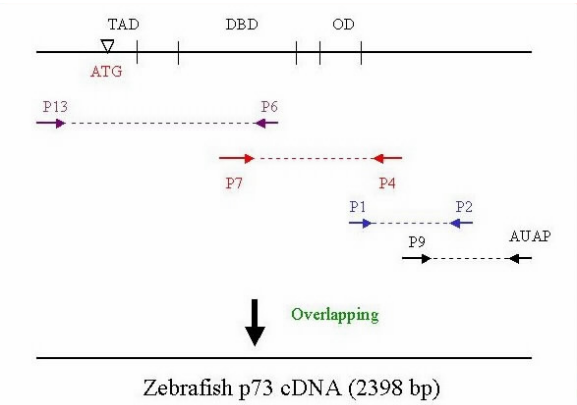

Fig.1 The strategy of cloning cDNA encoding a fragment of zebrafish p73. Primers used for PCR and RACE amplification are indicated.

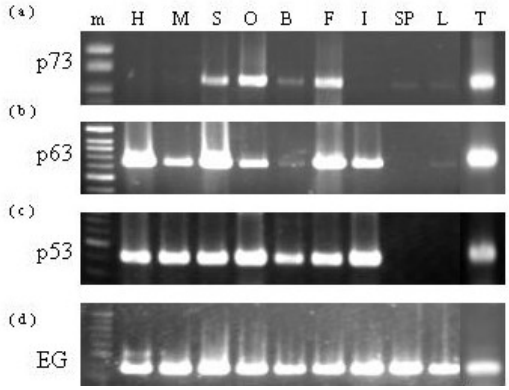

Fig.2 RT-PCR showing the expression of p73, p63, p53 and EG (elongation factorá1) in different adult tissues in zebrafish. EG served as an internal control. RNAs isolated from heart (H), muscle (M), skin (S), ovary (O), brain (B), fin (F), intestine (I), spleen (SP), liver (L)和 Testis (T) are assayed.

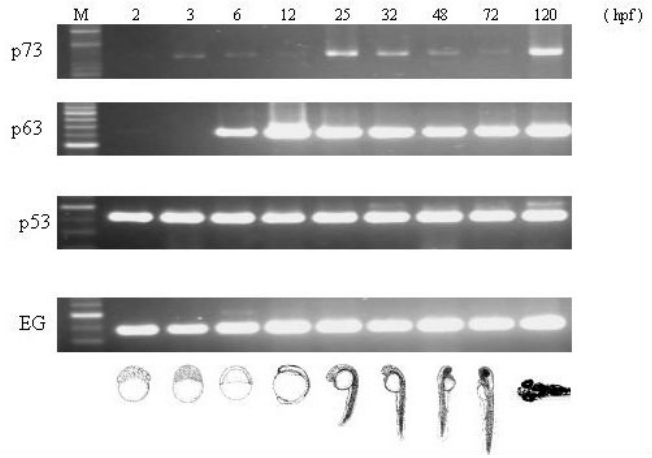

Fig.3 RT-PCR analysis of p53 family gene expression during different stages of embryogenesis in zebrafish. Hpf: hours post fertilization. EG (elongation factor á1) is used as an internal control.

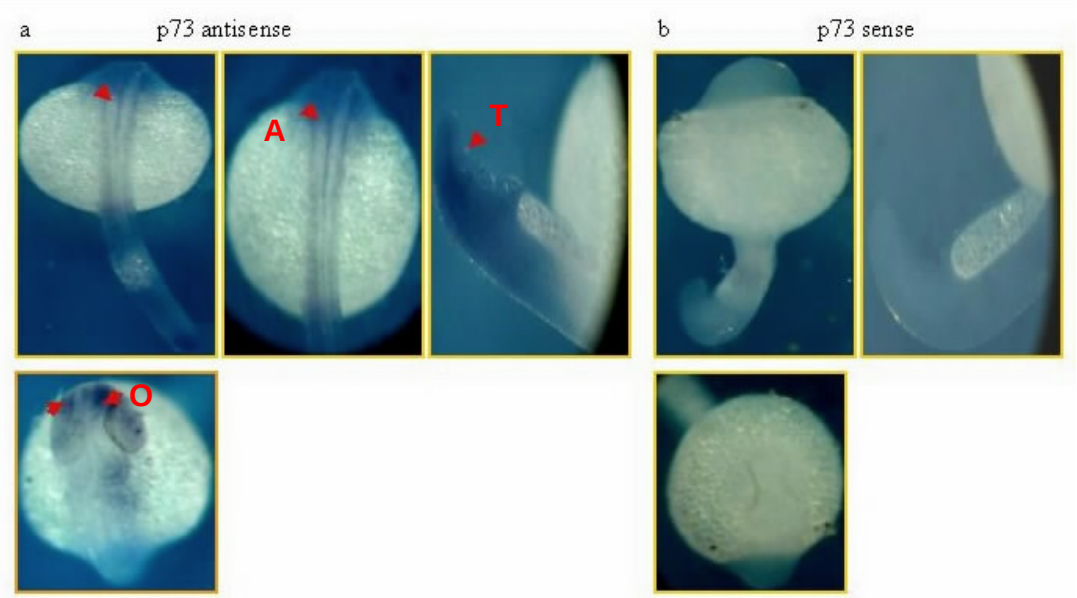

Fig. 4 Wholemount in situ hybridization of zebrafish embryo at 24 hpf. a, p73 antisense probe. b, p73 sense probe. A, artery. T, tail. O, olfactory system.

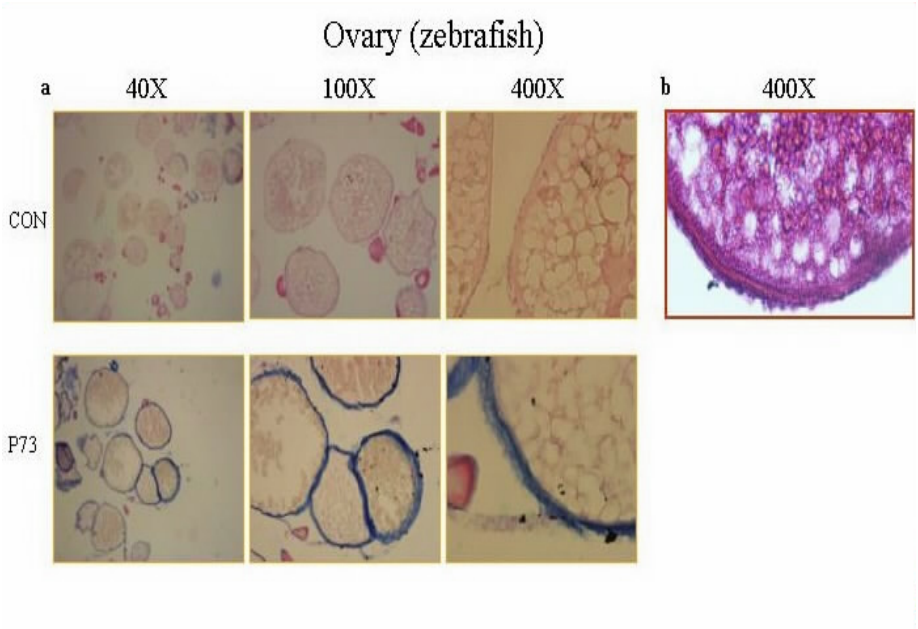

Fig. 5 Immunohistochemical staining of p73 α in zebrafish ovary. CON: control, without antibody; b, H & E staining. The positive stains are the granulose/theca cell layers.