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行政院國家科學委員會補助專題研究計畫成果報告

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※　野生型p53基因轉植對頭頸部腫瘤之基因治療探討　※

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計畫主持人：周 明 勇

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一、中文摘要

p53 基因突變普遍發生於口腔鱗狀細胞癌(oral squamous cell carcinoma, OSCC), 此一基因含約 20kb 的 DNA, 393 個氨基酸. 現今對於口腔鱗狀細胞癌的治療仍具相當數目的治療失敗情形.最近有研究指出 p53 腫瘤抑制基因(p53 tumor suppressor gene)可能關連於某些治療失敗的情形, 此一基因的不活化可能是現今與癌症治療效率有關的一個重要因子。於細胞培養中，惡性腫瘤之特性常可藉由植入正常的腫瘤抑制基因而致改善。正常野生型 p53(wild type p53,wt-p53)關聯於細胞週期進行。不正常或突變的 p53 基因可致突變基因積存及染色體重新排列。許多研究顯示，改正 p53 的異常有助於腫瘤的治療。於突變或缺失(deletion) p53 的人類肺癌細胞株及以裸鼠為模式的人類肺癌中植入野生型 p53 基因，可降低惡性腫瘤的表現。

Clayman 等人利用野生型p53基因於治療頭頸部腫瘤，結果顯示在體外(in vitro)野生型 p53 基因能阻止頭頸部鱗狀細胞癌(head and neck squamous cell carcinoma, HNSCC)細胞的增殖，且野生型 p53 蛋白質能誘發 HNSCC 細胞出現凋亡，其結果指出 p53 腫瘤抑制基因於治療頭頸部腫瘤具極佳的療效與較佳的應用前景。

為了評估野生型 p53 基因對頭頸部鱗狀細胞癌的基因療效，將經重組含野生型 p53 的腺病毒轉植於頭頸部鱗狀細胞癌的細胞株以探討其治療效率。本研究乃藉由現代分子生物學技術將野生型 p53 基因與腺病毒(adenovirus)載體(vector)構建重組體(recombinant)，經選殖重組的腺病毒液後將其轉染受體細胞，最後觀察經轉染的受體細胞對頭頸部鱗狀細胞癌於體內外的影響，進而對口腔癌的治療提供一條新的途徑。本計劃第一年為針對 TSCCA 及 GNM 二株癌細胞，對照正常細胞 BF、GF 及癌前細胞 OSF 之 p53 基因部份之變異，初步序列分析證實 p53 與口腔癌細胞之形成有相關聯性。

關鍵詞：頭頸部鱗狀細胞癌，野生型 p53 基因，腺病毒，基因治療，腫瘤抑制基因

Abstract

It is well established that a high incidence of p53 mutations exist in oral cavity squamous cell carcinomas. The p53 gene is contained within 20 kb of cellular DNA located on the short arm of human chromosome 17 at 17p13.1. Despite the combination of therapeutic options, there are still a large number of treatment failures and therefore major questions remain. Recent investigations suggest that mutations of the p53 tumour suppressor gene may account for some of the therapeutic failures. Inactivation of the gene may be an important determinant of the efficiency of today's multimodal therapy protocols. In cell culture, malignant properties can often be reversed by inserting normal tumor suppressor genes. Normal (wild-type) p53 is involved in the control of cell cycle progression as well as in arresting replication to permit repair in DNA damaged cells. It may also be involved in restricting precursor populations by mediating apoptosis or programmed cell death. Abnormal or mutant p53 permits the accumulation of gene mutations and chromosomal rearrangements.

There is experimental evidence showing the benefits derived from the correction of p53 abnormalities. Replacement of wild type p53 using adenoviral expression vectors in both human lung cancer cell lines with mutant or deleted p53 in vitro and in nude mouse model of orthotopic human lung cancer resulted in suppression of the malignant phenotype.

The aim of this study is to estimate the efficacy of the tumor suppressor gene wild-type p53 as single-agent gene therapy for squamous cell carcinoma of the head and neck (HNSCC). Recombinant cytomagalovirus (CMV) -promoted adenoviruses containing the wild-type p53 gene was transiently introduced into squamous cell carcinoma of the head and neck cell lines. This first year study has finished the comparison of p53 mutations of two oral cancer cell lines TSCCa and GNM, two normal cells BF and GF, and one precancer cells OSF. Preliminary sequencing data suggested the p53 mutations were correlated with the oncogenesis of oral cells.

Keywords: wild-type p53, tumor suppressor gene, HNSCC, gene therapy, adenovirus

二、緣由與目的

p53 is the most commonly mutated gene in human malignancy, prevalent in cancers of a wide variety of histogeneses and primary sites. This wide occurrence of defective p53 derives from 3 properties. First, wild type p53 is highly vulnerable to dysfunction caused by even a single base change in the coding sequence. Second, in contrast with classical tumor suppressor gene theory a single abnormal p53 allele or allele loss can alter phenotype. Depending on the gene lesion, this manifests by a gene-dose dependent reduction in certain p53 functions, a dominant negative inhibition of the remaining wild type allele's function, or gain of a novel function(s) not associated with wild type. Third, the participation of p53 in multiple pathways of fundamental importance to carcinogenesis makes it an Achilles' heel of cancer suppression, a defect in which can radically diminish cellular defences against carcinogenesis.

Developing gene therapy strategies may provide an opportunity for contemporary medicine to reasses the way solid malignant neoplasms are managed. Liu et al. previously demonstrated that the wild-type p53 adenovirus (Ad5CMV-p53) suppressed growth of established cell lines of squamous cell carcinoma of head and neck (HNSCC) (1). In a microscopic residual disease model that mimic the post-surgical environment of patients with advanced HNSCC, the introduction of exogenous wild-type p53 in an adenoviral vector prevented the growth of tumor cells implanted subcutaneously in nude mice (1). These effects were dependent on vector dose but independent on the tumor cell's endogenous p53 status, wild-type or mutated (2).

In head and neck of cancer, directly transferring genes to a microscopic residual carcinoma is not technically difficult. When the primary malignant neoplasma are removed, the tumor milieu is readily accessible for molecular therapy. Regional lymphatic dissection also provides access to the most likely site for residual or occult disease. Therefore, new means of addressing microscopic residual disease that use direct transfer of genes capable of specifically promoting tumor cell death and sparing nonmalignant cells may provide desperately needed improvement in local and regional control of these cancers (3). The importance of developing such strategies is emphasized by the fact that the overall survival rate of patients with HNSCC has remained essentially unchanged during the past 30 years. Other solid malignant neoplasms pose a similar treatment dilemma, and HNSCC may serve as a model, providing valuable information that can be translated to different systems (4).

Mutations of the p53 gene occur frequently in a variety of human malignancies. In experimental models, it has been shown that re-expression of wild-type p53 in tumors by DNA-mediated gene transfer suppressed tumorigenicity (5-7). Gotoh et al. and others demonstrated that recombinant adenovirus p53 gene (Ad-CMV-p53) inhibited prostate

cancer growth both in vitro and in vivo (8-10). The inhibition mediated by Ad-CMV-p53 is independent of the p53 status of the prostate cancer cell target(10). These genes function as cell-cycle regulators by controlling the activity of cyclin-dependent kinases (CDK) (11). Since the loss of growth control is a common feature of malignant transformation, it is reasonable to hypothesize that deranged CDK inhibitors will result in the loss of cell growth control and restoration or expression of CDK inhibitors, which will suppress tumor growth.

Adenoviruses were used for gene transfer to liver (19), heart (20), central nervous system (21), and cancer cells (4,14,22-26). Adenoviral vector in current use accommodates expression cassette up to 7.5 kb. Adenoviruses are large double-strand DNA virus which are highly efficient at transferring their DNA into a target cell. This process is mediated through endocytosis after adenovirus binds to a specific cell surface receptor. These vectors enter the cell by means of two receptors: a specific receptor for adenovirus fiber and $\alpha_v\beta_3$(or $\alpha_v\beta_5$) surface integrins that serve as a receptor for the adenovirus penton. Once inside the cell, the virus forms a pore in the endosome and releases the DNA, which translocates to the nucleus. In wild-type adenovirus this initiates activation of the viral genome and replication of adenovirus leading to the lysis of the cell. The adenoviruses used for gene therapy are rendered replication defective by removal of the early 1(E 1) region that is vital for transformation and viral trans-cription. Therefor, adenovirus with E1 deletions replicates only in host cells engineered to contain this region of the adenoviral genome. One such cell line, human embryonic kidney 293 cell, was transformed into an immortal line using the adenoviral E1 region. Deleting the genetic information from the adenoviral genome within this and other regions provides space within the virus to accompany more than 10 kb of foreign genetic material. A new recombinant adenovirus encoding a particular gene of interest is generated by first cloning the transgene into a plasmid incorporating an expression cassette and portions of the adenoviral genome. Recombination between the plasmid and the 'backbone' of the adenoviral genome result in a virus encoding the desired transgene (12-17).

三、結果與討論

<u>Detection of p53 mutations of TSCCa and GNM cell lines by PCR-dideoxyfingerprinting (ddF) sequence.</u>

PCR-ddF was used to detect the mutations of p53 in TSCCa and GNM cell lines. The ddF is a hybrid technique which combines aspects of SSCP and dideoxy sequencing and was developed to increase the sensitivity of mutation detection. In ddF, a DNA sequence of interest is subjected to PCR amplification and product purification.

Primers used for amplification of the human p53 cDNA fragment. Full-length cDNa of p53 was divided into 6 overlapping fragments. These fragments were modified to carry desired restriction sites, amplified via PCR and subcloned in-frame into plasmid pGEX-KG. Primer sequences used to amplify p53 cDNA fragments were summarized below.

Amplified fragment			Primers/Restriction sites	
Name	Nucleotde included	Lenght (bp)	Sequence	
P53-NS	1-213	213	5'-GGAGATATACCCATGGAGAGCCGCAG-3'	NcoI
			5'-GCCACGGGGGGAGCTCCCTCTGGCAT-3'	SacI
P53-SS	162-433	271	5'-TCACTGAAGCCCGGGTCCAGATGAA-3'	SmaI
			5'-AATCAACCCAGAGCTCCTAAGGGCAG-3'	SacI
P53-SE	383-566	173	5'-GCACGTACTCCCCGGGCCTCAACAG-3'	SmaI
			5'-TCCACACGCAGAATTCCTTACACTCG-3'	EcoR
P53-XH	561-786	225	5'-ATAGCGATGGTCTAGACCTTACACTCG-3'	XbaI
			5'-TCCACACGCAGAATTCCTTACACTCG-3'	HindIII
P53-NH	749-984	235	5'-AACCGGAGGCCCATGGTCACCATC-3'	NcoI
			5'-CACGGATCTGAAGCTTGAATAATTGT-3'	HindIII
P53-BX	936-1181	245	5'-CAACAACCACCGGATCCTCTCCCCAGC-3'	BamHI
			5'-AGCAGCCAACTAAGCTTCCT-3'	HindIII

四、計畫成果自評

This first year study has finished the comparison of p53 mutations of two oral cancer cell lines TSCCa and GNM, two normal cells BF and GF, and one precancer cells OSF. Preliminary sequencing data suggested the p53 mutations were correlated with the oncogenesis of oral cells. For second year, achieving viral gene transfer to specific organs for clinical application will be difficult; however, particularly as viral titers 10 to 1000 times higher than those usually attained (typically 10^6 infectious units per ml) will be necessary for in vivo strategies(18). Adenovirus vectors are well suited for in vivo transfer applications because they can be produced in high titers(up to 10^{13} viral particles/ml) and they transfer genes efficiently to non-replicating and replicating cells. The transferred genetic information remains epichromosomal, thus avoiding the risks of permanently altering the cellular genotype or insertional mutagenesis (12).

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