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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※ **早老症分子機轉之研究** ※
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計畫主持人：胡務亮
共同主持人：

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行政院國家科學委員會專題研究計畫成果報告

早老症分子機轉之研究

A study on the molecular basis of progeria

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一、中文摘要

老化是一個奧祕的過程，由於對於老化的研究似乎一時很難得到清楚的結論，因此有人認為早老症候群的原因及機轉比較單純，應是研究老化的一種適合對象。早老症(Hutchinson-Gilford 氏症)是一種極為罕見的疾病，患者生長極度不良，嬰幼兒期就出現一些老年人的特徵，如皮膚萎縮、禿頭、皮下脂肪消失、頭皮血管突出、四肢瘦小及骨骼吸收等。患者早期出現動脈硬化，通常十幾歲以前就死於心臟血管疾病。早老症的原因目前還不清楚。而大部分其他的早老症候群目前發現多半和基因修補或複製的缺損有關，比如 Werner syndrome 的 WRN 基因是一個 DNA helicase 的蛋白質。這些疾病表現出相當明顯的紫外線敏感性、基因缺損及腫瘤等現象。

過去關於早老症的研究發現，包括皮膚第四型膠原纖維及彈性纖維增高、醣蛋白 GP200 提高、基因修補控制異常等，缺乏特異性。為了突破研究上的困境，我們決定以 differential display 試圖去了解早老症的分子機轉。我們已有培養多株本土性早老症皮膚纖維芽細胞，計劃以 differential display 的技術，可以對早老症的病因做一些推斷。我們先抽取正常皮膚纖維芽細胞以及早老症患者皮膚纖維芽細胞中之 RNA。所選取的細胞大約是 10 自 12 代，此時兩種細胞的外型還沒有差異。早老症的細胞大約只能分裂至 20 代左右，而正常細胞常常可以到 30 代。我們將 RNA 以非放射線法標誌後，與 micro-array 濾紙雜合。不過結果兩種細胞的差異相當大，而且背景的雜訊也相當高。我們經過多次努力仍然無法克服這個問題，另外實驗室之設備也無法進行放射線元素之實驗，因此到目前為止還沒有具體之成果產生。未來如果有機會，將使用新一代的 micro-array，由雜合至顯像分析完全自動化，或可克服目前的困難。

關鍵詞：早老症， Hutchinson-Gilford **氏症，** differential display

Abstract

Aging is a mysterious process results from multiple factors, and studies of the causes of aging are often obscured by the complexity of this phenomenon. Therefore, it is suggested that progeria, which presents a less complicated etiology and phenotype may allow research to focus on a regulation site involved in development and aging. Hutchinson-Gilford progeria syndrome (progeria) is a rare disease characterized by selected features of premature aging. Presentations include a general failure of the child to thrive, and features of an aged appearance of the skin, alopecia, decreased subcutaneous fat, prominent scalp veins, thin limbs, and osteolysis. The formation of atherosclerotic plaques result in the death of the majority of progeria patients in the second decade of life. The etiology of progeria is still unknown. Some other progeroid syndromes have been found to be associated with defects in either DNA repair or replication. For example, the Werner syndrome WRN gene is a DNA helicase. These diseases usually demonstrate clear UV sensitivity or tumor formation.

Previous studies on progeria showed many non-specific changes. In order to make a breakthrough the molecular basis of progeria, we plan to do differential display study. We have had cultured skin fibroblasts from domestic patients with progeria. We have extract RNA from skin fibroblasts from both normal controls and progeria patients. The passage numbers are from 10 to 12. Ordinarily the progeria cells survive not much beyond 20 generations, but normal cells usually survive more than 30 generations. We label the RNA with non-isotope method. The labeled probes were used to hybridize micro-array filter paper. The results showed that the differences between the two kinds of cells were quite large, and the background noise was high. We tried several times, but we still can not get readable result to further analysis. We hope in the future, modern micro-array techniques could be applied to this project, that both hybridization, signal development and reading will be automatic. Then we will have chance to overcome current obstacles.

Keywords: Progeria, Hutchinson-Gilford syndrome, differential display

二、緣由與目的

Premature aging

Aging is a mysterious process result from a combination of genetic, environmental and social factors. Although the biological study of aging has

recently been making rapid progress, studies of the causes of aging are often obscured by the complexity of this phenomenon. Therefore, it has been proposed that to gain insight into aging, the problem may first be simplified by restricting studies to an aspect of the aging process, the accelerated aging diseases. Several rare conditions exist in man and exhibit certain phenotypic characteristics associated with premature ageing. Often referred to as progeroid syndromes, including Hutchinson-Gilford syndrome, Werner's syndrome, Cockayne's syndrome, Bloom syndrrome, ataxia telangiectasia and Down's syndrome. (Martin GM, 1989)

Hutchinson-Gilford progeria syndrome (progeria) is a rare disease characterized by selected features of accelerated and premature aging (Debusk FL, 1972; Beauregard and Gilchrest, 1987). Initial presentations involve cutaneous abnormalities and a general failure of the child to thrive. Diagnosis of progeria is usually made in the first year of life. By the end of the child's second year, typical features of progeria become apparent. These include an aged appearance of the skin (often sclerodermatous on the phalanges, and thin and taut on the face), alopecia, decreased subcutaneous fat, prominent scalp veins and thin limbs (Badame, 1989). Other abnormalities involve the skeletal system and include prominent joints, osteoporosis and osteolysis. The formation of atherosclerotic plaques and other perturbations of the cardiovascular system result in the death of the majority (approximately 75%) of progeria patients from myocardial infarction, usually in the second decade of life (Goldstein, 1971). There are similar biochemical or physiological abnormalities between patients with progeria syndrome and Werner's syndrome. But the onset or death age is earlier (Jones KL, 1997). No evidence presents that progeria syndrome is related to the WRN gene (Oshima J, 1996). The real reason of this early onset progeria is still unclear.

In progeria and Werner's syndrrome, most biochemical abnormalities are associated with connective tissue, principally of mesodermal origin. The most thoroughly investigated is the finding of 10-20 times greater urinary excretion of hyaluronic acid in the patients with progeria compared with controls (Zebrower M, 1986; Badame, 1989). Similar results have also been obtained in the patients with Werner's syndrome (Goto M, 1978). There are other abnormalities found in progeria. Gene expression in skin fibroblasts from progeria patients was abnormal, where collagen type IV and elastin mRNA was elevated (Colige et al., 1991), the over-expression of a 200kDa glycoprotein (Clark and Weiss, 1993).

Cultured fibroblasts from the patients with progeria and Werner's syndrome showed a markedly reduced potential for in vitro growth and have a shorter replicative life span compared with control cultures (Martin GM, 1970). More studies have been done in Werner syndrome, especially triggered by the cloning of the WRN gene. The cell cycle defect in Werner syndrome may be due to impaired S-phase transit because of prolongation of S phase in the cell cycle (Poot M, 1992) and decreased expression of regulators, such as platelet-derived growth factor (PDGF) β-receptors (Mori S, 1993). But there is no comparable study in progeria.

In order to overcome these difficulties, we plan to take advantage of current advance in genome research, the differential display technique. This technique will give us a general idea about the changes in progeria cells. It is already known that c-myc expression in progeria fibroblasts was elevated (Nakamura et al., 1988). However, we have to see a pattern in order to formulate hypothesis about the etiology of progeria. Differential display will give the overall picture at one shot. However, many changes including those associated with normal aging process will also be discovered. We have to compare the progeria cells with normal cells with either the same passage number or the same old. Other progeroid cells, including from Cockayne syndrome, will be another kind of control. Nevertheless, we don't intended to get the progeria gene only by this technique.

三、方法，結果與討論

In vitro culture of normal and progeria fibroblasts

Progeria fibroblasts came from two patients, one 5 years old (case 1) and the other 1 year old (case 2). Although they are both young, clinical manifestations are clear. They (the younger patient at the age of 2) had alopecia, prominent scalp veins, typical facial appearance, absorption of clavicle, atrophic skin, and hypertrophic muscle. They both very small, but have normal mental development. Cardiac function and echocardiogram were normal. Control fibroblast was cultured from a 5-year-old boy with unrelated disease. Cells will be split at a 1 to 3 ratio. Cells will be collected for either DNA or RNA/protein alternatively since p5. The growth of cells from case 1 slowed down since p15, and completely stop grow at p18. The control fibroblasts kept grow till p29. One control fibroblast transformed spontaneous at p25. The morphology of the cells become polygonal with occasional clumping. Chromosome analysis showed aneuploidity However, the speed of growth of these transformed cells slowed down after around another 20 splits. Therefore, these cells may not pass the "crisis" of immortalization (Harley and Villeponteau, 1995).

Preparation of RNA

Total RNA is isolated by the Atlas Pure Total RNA Labeling System (# K1038-1).

Preparation of cDNA probe

Labeling of RNA by a non-isotope method was done following the kit accompanying the Atlas Array kit.

Hybridization Procedure

Test Hybridization to blank membrane

Prepare sheared salmon testes DNA in ExpressHyp solution

1.Denature sheared salmon testes DNA at 95-100°C for 5 minutes and chill on ice quickly.
2.Keep the denatured sheared salmon testes DNA with pre-warmed ExpressHyb at 68 °C till use.

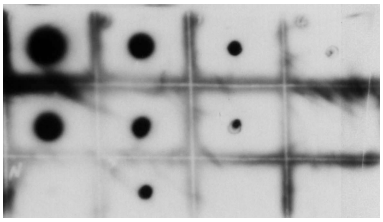


Figure: test hybridization

Hybridizing cDNA probes to the Atlas Array

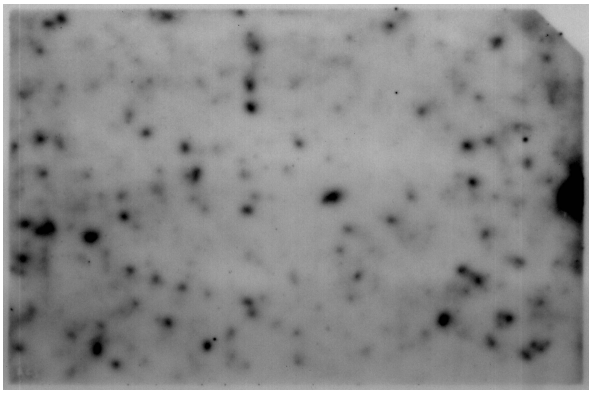
a) Prepare the Atlas Array for prehybridization
1.Place the membrane in the 200ml pre-warmed 0.5% SDS solution for 2 minutes.
2.Transfer the membrane to hybridization bottle.
3.Rinse the membrane with deionized H_2O.

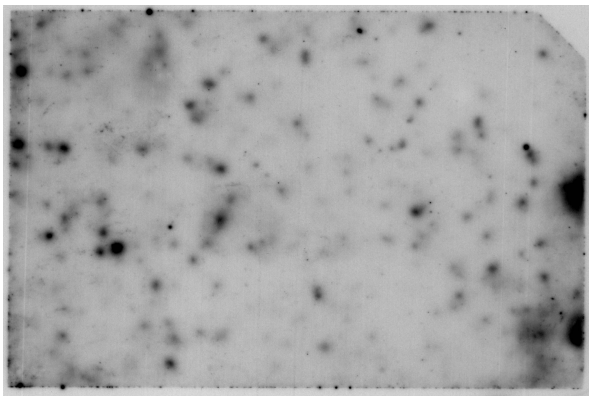
b) Prehybridization
1. Prehybridize in 5ml sheared salmon testes DNA solution with continuous agitation at 68 °C for 30 minutes.

c) Hybridization
1.Prepare the probe by boiling for 2 minutes and chilling on ice for 2 minutes.
2.Pour the probe carefully and directly on the surface of the membrane.
3.Mix well.
4.Hybridize overnight with continuous agitation at 68 °C.
5Wash the membrane with 2X SSC for 30 minutes with continuous agitation at 68 °C 3 times.
6.Wash the membrane with 1% SDS and 0.1X SSC, 0.5% SDS for 30 minutes with continuous agitation at 68 °C.
7.Wash the membrane with2X SSC with continuous agitation at RT.
8.Wrap the damp membrane in a plastic wrap immediately.
9.Mount the plastic-wrapped Atlas Array on 3mm Whatman paper.
10.Expose the Atlas Array to X-ray film at -70 °C.

Selection of microarray

Our first selection of Atlas array would be a generalized Human cDNA array (588 clones), other arrays considered would be Human Apoptosis, Human Cancer, Human Cell Cycle, Human Cell Interaction, Human Cytokine/Receptor, Human Oncogene/Tumor Suppressor etc. Microarray from other company with more clones but less expensive will also be considered.





The results showed that the differences between the two kinds of cells were quite large, and the background noise was high. We tried several times, but we still can not get readable result to further analysis. We hope in the future, modern micro-array techniques could be applied to this project, that both hybridization, signal development and reading will be automatic. Then we will have chance to overcome current obstacles.

四，參考文獻

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 89, 10114-10118.

Badame AJ (1989) Progeria. Arch Dermatol 125, 540-544.

Beauregard S, Gilchrest BA (1987) Syndromes of premature aging. Dermatologic Clinics 5, 109-121.

Bohr VA, Anson RM (1995) DNA damage, mutation and fine structure DNA repair in aging. Mut Res 338, 25-34.

Clark MA, Weiss AS (1993) Elevated levels of glycoprotein gp200 in progeria fibroblasts. Mole Cell Biochem 120, 51-60.

Colige A, Roujeau JC, De La Rocque F, Nusgens B, Lapiere CM (1991) abnormal gene expression in skin fibroblasts from a Hutchinson-Gilford patient. Lab Invest 64, 799-806.

Ellis NA (1997) DNA helicase in inherited human

disorders. Current Opinion in Genet Develop 7, 354-363.
Debusk FL (1972) The Hutchinson-Gilford progeria syndrome. J Pediatr 80, 697-724.)
Goldstein S (1971) The biology of aging. New Eng J Med 285, 1120-1128.
Goto M and Murata K (1978) Urinary secretion of macromolecular acidic glycosaminoglycans in Werner's syndrome. Clin. Chim. Acta 85, 101-106.Gray MD, Shen JC, Ashwini SKL, Blank A, Sopher BL, Martin GM, Oshima J and Loeb LA (1997) The Werner's syndrome protein is a DNA helicase. Nature Genetics 17, 100-103.
Gupta RS (1980) Senescence of cultured human diploid fibroblasts. Are mutations responsible? J Cell Physiol 103, 209-216.
Harley CB, Villeponteau B (1995) Telomeres and telomerase in aging and cancer. Curr Opionion Genet Develop 5, 249-255.
Jones KL (1997) Smith's recognizable patterns of human malformation: Progeria syndrome. 5th.edition, 138-143.
Kodama S, kashino G, Suzuki K, Takatsuji T, Okumura Y, Oshimura M, Watanabe M, Barrett JC (1998) Failure to complement abnormal phenotypes of Simian virus 40-transformed Werner syndrome cells by introduction of a normal human chromosome 8. Cancer Res 58, 5188-5195.
Lee HC, Wei YH (1997) Mutation and oxidative damage of mitochondrial DNA and defective turnover of mitochondria in human aging. J Formos Med Assoc 96, 770-778.
Lipman JM, Applegate-Stevens A, Soyka LA, Hart RW (1989) Cell-cycle defect of DNA repair in progeria skin fibroblasts. Mut Res 219, 273-281.
Martin GM (1989) Genetic modulation of the senescent phenotype in homo sapients. Genome 31, 390-397.
Mills RG, Weiss AS (1990) Does progeria provide the best model of accelerated aging in humans? Gerontology 36, 84-98.
Mori S, Kawano M, Kanzaki T, Morisaki N, Saito Y and Yoshida S (1993) Decreased expression of the platelet derived growth factor beta-receptor in fibroblasts from a patient with Werner's syndrome. Eur. J. Clin. Invest. 23, 161-165.
Munscher C, Rieger T, Muller-Hocker J, et al (19943) The point mutation of mitochondrial DNA characteristic for MERRF disease is found also in healthy people of different ages. FEBS lett 317, 27-30.
Nakamura KD, Turturro A, Hart RW (1988) Elevated c-myc expression in progeria fibroblasts. Biochem Biophys Res Comm 155, 996-1000.
Narendra T and Renu T (1996) DNA helicases: the long unwinding road. Nature Genetics 13, 11-12.
O'Brien ME, Weiss AS (1991) Hutchinson-Gilford progeria fibroblasts exhibit metabolically normal uridine uptake and RNA synthetic rates. Biochem Biophys Res Comm 210, 225-230.
Oshima J, Brown WT, and Martin GM (1996) No detectable mutations at the Werner's helicase locus in progeria. The Lancet 348, 1106.
Poot M, Hoehn H, Runger TM and Martin GM (1992) Impaired S-phase transit of Werner's syndrome cells expressed in lymphoblastoid cell lines. Exp. Cell Res. 202, 267-273.
Poot M, Gollahon KA, Rabinovitch PS (1999) Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S-phase. Hum Genet 104, 10-14.
Prince PR, Qgburn CE, Mose MJ, Emond MJ, Martin GM, Monnat RJ Jr (1999) Cell fusion corrects the 4-nitroquinoline 1-oxide sensitivity of Werner syndrome fibroblast cell line. Hum Genet 105, 132-138.
Salk D (1982) Werner's syndrome: A review of recent research with an analysis of connective tissue metabolism, growth control of cultured cells, and chromosome aberrations. Human Genetics 62, 1-5.
Suzuki N, Shimamoto A, Imamura O, Kuromitsu J, Kitao S, Goto M and Furuichi Y (1997) DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. Nucleic Acids Research 25, 2973-2978.
Tanaka K, Nakazawa T, Okada Y, Kumahara Y (1979) Increase in DNA synthesis in Werner’s syndrome cells by hybridization with normal human diploid and HeLa cells. Exp Cell Res 123, 261-267.
Torroni A. Petrozzi M. D'Urbano L. Sellitto D. Zeviani M. Carrara F. Carducci C. Leuzzi V. Carelli V. Barboni P. De Negri A. Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. Am J of Hum Genet 60, 1107-1121.
Wang S, Nishigori C, Yagi T, Takebe H (1991) Reduced DNA repair in progeria cells and effects of r-ray irradiation on UV-induced unscheduled DNA synthesis in normal and progeria cells. Mut Res 256, 59-66,
Wei YH, Pang CY, You BJ, et al (1996) Tandem duplications and large-scale deletions of mitochondrial FNA are early molecular events of human aging process. Ann NY Acad Sci 786, 82-101.
Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J, Schellenberg GD (1996) Positional cloning of the Werner's syndrome gene. Science 272, 258-262.
Zebrower M, Kieras FJ and Brown WT (1986) Urinary hyalurronic acid elevation in Hutchinson-Gilford progeria syndrome. Mech. Ageing Dev. 35, 39-46.
Zhang C, Linnane AW, Nagley P (1993) Occurrence of a particular base substitution (3242A to G) in mitochondrial DNA of tissues of aging humans. Biochem Biophys Res Commun 195, 1104-1110