國科會專題研究計畫成果報告

骨髓形成不良症候群多重腫瘤抑制基因過度甲基化的研究，第一年

Hypermethylation of Multiple Suppressor Gene in Myelodysplastic Syndrome, The First Year

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一、計畫中文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

關鍵詞：多重腫瘤抑制基因、過度甲基化、骨髓形成不良症候群

多重腫瘤抑制基因 (multiple tumor suppressor gene, MTS) 1/p16^{INK4A}及 MTS2/p15^{INK4B}位於染色體 9p21 上，他們的蛋白產物可經由抑制 cyclin-dependent kinase 4 及 6 而阻止細胞週期由 G1 進入 S 期，爲負向調控基因；同時這兩個基因在許多腫瘤細胞株或原始腫瘤組織中被發現有 homozygous deletion，所以被認爲是 tumor suppressor genes, 與腫瘤的發生有關。在我們過去的研究中，發現 T-淋巴芽細胞白血病有不少病人有 p16 gene deletion，其中約一半同時有 p15 gene deletion，但骨髓形成不良症候群(簡稱 MDS)及急性骨髓性白血病均未發現有其中任一基因之 deletion。最近的研究顯示除了 deletion 外，此二基因 promotor 處 CpG islands 之過度甲基化 (hypermethylation)，也可造成基因的去活性。部分急性骨髓性及淋巴芽細胞性白血病人被發現有 p15 基因，但非 p16 基因，的過度甲基化；而淋巴瘤病人則多爲 p16 基因的過度甲基化。關於 MDS, 目前只有少數論文及摘要，指出此種病人亦常有 p15 基因的過度甲基化，可能與 MDS 的發生及進行有關。MDS 爲一同源性造血幹細胞疾患，病人一般會出現周邊血液全血球減少，但骨髓卻異常增生。其發生機轉尚不清楚，染色體及基因異常爲可能原因。由於目前對 MDS p15/p16 基因過度甲基化的報告有限，病人數目不多，且與疾病預後的關係尚未釐清，我們計畫對 MDS 病人發病時及追蹤過程中一系列的骨髓細胞，進行 p15/p16 基因甲基化的研究，探索其在 MDS 各亞型的發生率，及其與疾病進行、轉化及預後間的關聯性，以提供給臨床醫師作爲治療病人的參考。最近 Decitabin, 一種具有去甲基化作用的藥物，被發現可治療 MDS，初步反應不錯，但還有待進一步的臨床試驗；p15/p16 基因甲基化研究的結果，或許有助於日後判斷何種病人適合接受此種藥物的治療。

二、計畫英文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

Keywords：MTS1/p16^{INK4A}, MTS2/p15^{INK4B}, hypermethylation, myelodysplastic syndrome

Multiple tumor suppressor gene 1 (MTS1) / p16^{INK4A} and MTS2 /p15^{INK4B} were localized to chromosome 9q21. Their protein products negatively regulate the progression of cell cycle from G1 to S phase by inhibition of cyclin-dependent kinase 4 and 6, and have been considered as tumor suppressor genes. Deletion of the genes has been detected in many cancer cell lines or primary tumor tissues. In our previous study, we found that 47% of patients with T-acute lymphoblastic leukemia (ALL) had homozygous deletion of p16 gene and half of them had homozygous deletion of p15 gene simultaneously. None of patients with acute myeloid leukemia (AML) or myelodysplastic syndrome showed either of the gene deletion. An alternative mechanism of gene inactivation is hypermethylation at CpG islands within the promotor region of the genes. Recently, hypermethylation of p15 gene was observed in AML and ALL and that of p16 gene in non-Hodgkin's lymphoma. Only few studies concerning methylation state of p15/p16 genes in MDS has been reported, in which hypermethylation of p15 gene, but not p16, was detected frequently in the disease. MDS is a clonal disorder of hematopoietic stem cells. The pathogenesis of the disease is largely unknown. Clonal chromosomal abnormalities and gene alternation may play important roles in the carcinogenesis. In this study, we will analyse methylation states of p15/p16 genes on bone marrow cells from patients with MDS at diagnosis and during serial follow-up. The prognostic implications will be clarified in the study. Recently, decitabine, a hypomethylation agent, has been applied to the treatment of MDS with good result. The study of methylation states of p15/p16 genes may help to identify the proper candidates for decitabine treatment.

三、研究計畫之背景及目的：

The multiple tumor suppressor gene 1 (MTS1)/p16^{INK4A} and MTS2/p15^{INK4B} genes encode proteins negatively regulating the cell cycle by inhibition of cyclin-dependent kinase 4 and 6, which control progression of cells from G1 to S phase[1,2]. Both genes were localized to chromosome 9p21, a locus frequently found to be deleted in cancer cells, and was considered as candidate tumor suppressor genes[3-6]. Homozygous deletion of p15 and p16 genes has been detected in many cancer cell lines and primary tumors[4,7,8]. In a previous study, we demonstrated that 47% of patients with T-lymphoblastic leukemia (ALL) had homozygous deletion of p16 gene and half of them showed homozygous deletion of p15 gene simultaneously[9]. None of patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) had deletion of either of the two genes (unpublished data). These findings were compatible with those reported in literature that p16/p15 gene deletion usually occurred in lymphoid malignancies, but rarely in myeloid malignancies[10-12].

An alternative mechanism of gene inactivation is hypermethylation of CpG islands within the promotor region of the genes[13,14]. These GC-rich regions are generally unmethylated in normal tissues, and their methylation is associated with transcriptional loss[15]. Recent studies have shown that hypermethylation of p15 gene, but not p16 gene, was observed in AML and ALL[16]. On the other hand, the p16 gene was frequently hypermethylated in non-Hodgkin's lymphoma[16].

MDS is a clonal hematopoietic disorder characterized by pancytopenia in peripheral blood despite a hypercellular bone marrow(BM). Evolution to acute leukemia occurs in about 10% to 35% of such cases[17-19]. According to French-American-British classification, five subgroups can be found: refractory anemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMOL)[20]. The abnormalities seen in MDS have been ascribed to deregulation

of cell proliferation, differentiation, or apoptosis, but the pathogenesis of the disease is largely unknown. We have demonstrated that half of MDS patients had clonal chromosomal abnormalities and 20% showed N-ras mutation[19]. Both are thought to play important roles in carcinogenesis. Patients with complex cytogenetic abnormalities had higher risk for developing acute leukemia and had a shorter survival than other patients. Recently, Uchida et al reported that hypermethylation of p15 gene, but not p16 gene, occurred frequently in MDS patients and suggested it might be involved in the pathogenesis of the disease[21]. The patients number in that study was limited with only a few patients being serially follwed up and the prognostic implications of p15 hypermethylation in MDS was not well explored. In this study, bone marrow cells from MDS patients at different stages of the disease will be serially analysed for methylation states of p15/p16 genes. The results will be correlated with subtypes of MDS, cytogenetic results, progression to leukemia and survival of the patients.

After we applied to the National Science Council for a grant for this study in January, 1998, a second paper concerning p15/p16 hypermethylation in MDS was published[22] which showed similar results as those reported by Uchida et al[21]. Recently, decitabine (DAC), a hypomethylation agent, has been used for the treatment of MDS with good result[23,24]. More clinical trials are needed to confirm the finding. In the Annual Meeting of American Society of Hematology in December, 1998, Daskalakis et al reported that the fraquency of p15 gene hypermethylation was decreased following DAC treatment[25]. The study of methylation states of p15/p16 genes may help to find the proper candidates for DAC treatment in the future.

In the first year of the studies, we cloned the exon 1 of p15 and p16 gene, respectively, which were used as probes in Southern blotting analysis for methylation states of the two genes. The specificity of the probes was confirmed by sequencing. The preliminary results of the Southern blotting analysis were shown as follows:

四、第一年研究初步成果：

1.BM cells from 33 (65%) of the 51 patients with MDS showed hypermethylation of p15 gene by Southern blot analysis, but none of the 20 patients studied had hypermethylation of p16 gene.

2.Serial studies of methylation states of p15 gene have been performed on two patients. One showed progressive increase of methylation of p15 gene during the follow-up (Fig 1a), while the other showed disappearance of methylation after a successful bone marrow transplantation (Fig1b).

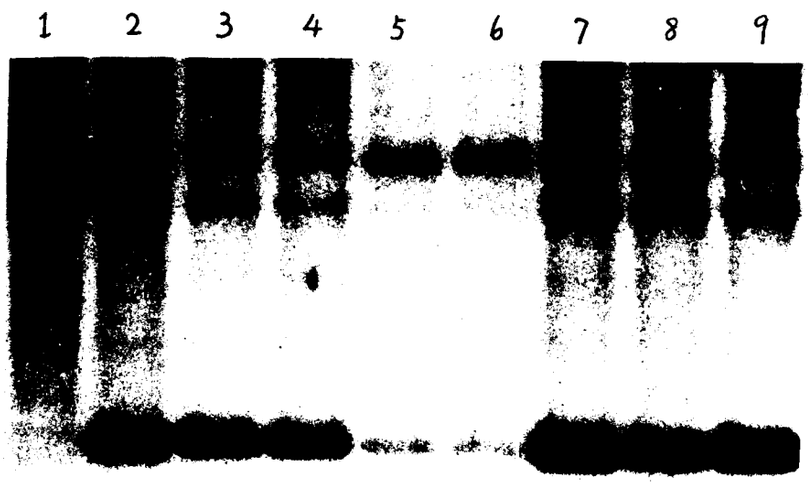

Fig 1a Southern blotting of p15 gene. Lanes 1 & 2 are control DNA, lanes 3-6 are DNA from a MDS patient (case 1) at diagnosis and subsequent follow-up, and lanes 7,8,9 are from three other patients. DNA is digested with Hind III alone in lane 1, with both Hind III and Eag I (methylation sensitive restriction enzyme) in other lanes. Unmethylated pattern is seen in lanes 1 & 7, incomplete methylation in lanes 3,4,8, & 9 and complete methylation in lanes 5 & 6.

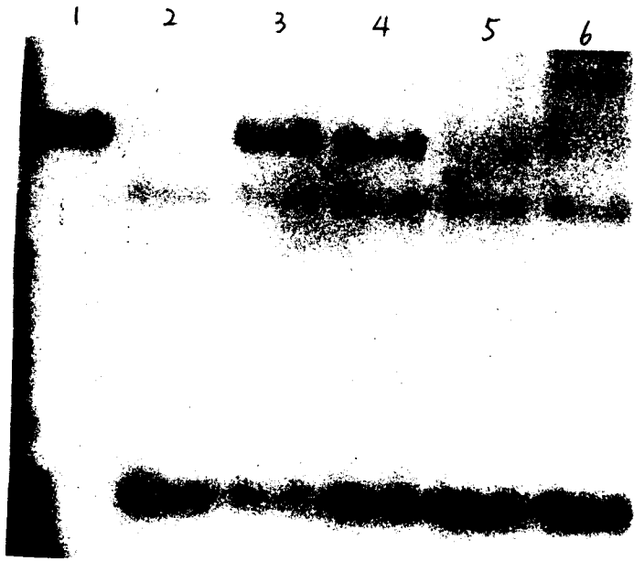

Fig 1b Southern blotting of p15 gene. Lanes 1 & 2 are control DNA, lanes 3 to 6 are DNA from a MDS patient (case 2) at diagnosis (lane 3), before allogeneic bone marrow transplantation (BMT)(lane 4) and after BMT (lanes 5 & 6). It shows hypermethylation of p15 gene in lanes 3 & 4 from case 2 before BMT and unmethylated pattern in lane 2 (control) and lanes 5 & 6 from case 2 after BMT.

In the second and third year of studies, we like to recruit more patients for analysis, with special emphasis on serial studies of individual patients. To exclude the possibility of incomplete digestion by methylation sensitive restriction enzyme EagI, which may give a false positive result, all samples showing methylated patterns will be examined at least twice by Southern blot analysis. To make the data more reliable, methylation-specific PCR[25], which is a more specific and sensitive technique, will be set up and applied to the study (please see the method). Correlation of the result with FAB subtype of MDS, progression of the disease and prognosis of the patients will also be analysed.

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