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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※ 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 ※
※ Hypermethylation of Multiple Suppressor Gene in ※
※ Myelodysplastic Syndrome ※
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計畫參與人員: 沈銘鏡、陳耀昌、蔡偉、唐季祿、姚明

一、中英文摘要

為了要了解骨髓增生不良症候群(簡稱 MDS) p15 基因(又稱為多重腫瘤抑癌基因 2)甲基化的異常，及其與疾病轉化為白血病及病人存活間的關係，我們對 50 位 MDS 病人，系列追蹤其骨髓細胞 p15 基因啟動區域甲基化的情形。p15 基因的甲基化，會造成此基因的去活化，被認為與腫瘤的發生可能有關。50 位病人中，共有 17 位(34%)呈現 p15 基因甲基化的現象，有些病人在診斷時即有此變化(7/43,16%)，有些是在追蹤過程中出現。根據國際 FAB 分類法，MDS 可分為 refractory anemia (RA)，RA with ring sideroblasts (RARS)，RA with excess of blasts(RAEB)，RAEB in transformation(RAEB-T) 及 chronic myelomonocytic leukemia(CMML)，五類，前二類屬低危險 MDS，後三類屬高危險 MDS。低危險 MDS 發生 p15 基因甲基化的機率為 0%，在追蹤過程中若疾病沒有進行至高危險 MDS，p15 基因亦維持在非甲基化狀態；高危險 MDS 在診斷時 p15 甲基化的發生率為 23%，追蹤過程中若無進級至更嚴重一類(如 RAEB→BAEB-T) 或轉為急性白血病，則甲基化的機率差不多，為 30%。初轉化為急性骨髓性白血病(AML)者，初期 p15 甲基化的發生率為 60%，最後會再增高至 75%。發生 p15 甲基化的病人中，69%最後會轉化為 AML，反之無 p15 甲基化病人，僅 22%發生 AML。在單變數分析中，p15 甲基化與病人存活較短有關，但在多變數分析中，僅有 FAB 分類及染色體變化與存活有關。
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

Abstract

To investigate the time sequence of occurrence of $p15^{INK4B}$ gene methylation in myelodysplastic syndrome (MDS) and its correlation with leukaemic transformation and survival of patients, methylation status of the $p15^{INK4B}$ promoter region was analyzed on 50 patients, and was serially studied on 22 of them. Overall, 17 (34%) of the 50 patients showed $p15^{INK4B}$ gene methylation. It could first be demonstrated at diagnosis or during follow-up. When FAB subtypes at the time of study were used in analysis, the incidence of $p15^{INK4B}$ methylation in each risk group of MDS remained stable through the course: 0% through for low-risk MDS (refractory anemia [RA] and RA with ring sideroblasts), and from 23% at diagnosis to 30% finally for high-risk MDS (RA with excess of blasts [RAEB], RAEB in transformation and chronic myelomonocytic leukemia), respectively. The incidence of $p15^{INK4B}$ methylation raised to 60% at initial study and to 75% finally in acute myeloid leukaemia (AML) evolved from MDS. Most patients (69%) with $p15^{INK4B}$ methylation showed disease progression to AML; it could be

detected before, at the time of, or after the diagnosis of leukaemic transformation. $p15^{INK4B}$ methylation in MDS patients implicated a shorter survival time in univariate analysis, but its prognostic significance disappeared in multivariate analysis. In conclusion, $p15^{INK4B}$ methylation can be detected early at diagnosis of MDS or acquired during disease progression. It may play an important role in the pathogenesis of some high-risk MDS and is related to leukaemic transformation of MDS.
Key words: $p15^{INK4B}$, myelodysplastics syndrome, and methylation

二、研究計畫之背景及目的

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by peripheral blood cytopenia due to ineffective hematopoiesis (Galton *et al*, 1986). Evolution to acute myeloid leukaemia (AML) occurs in about 10% to 35% of such cases (Geddes *et al*, 1990; Jacobs *et al*, 1986; Tien *et al*, 1994; Yunis *et al*, 1986). Five subgroups of MDS can be identified according to the French -American-British (FAB) classification which is based on the cell morphology and the percentage of blast cells in the peripheral blood or bone marrow (Bennett *et al*, 1982) : refractory anemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukaemia (CMML). Although clonal chromosomal abnormalities, which can be demonstrated in about 50% of patients, are well characterized in MDS (Jacobs *et al*, 1986; Tien *et al*, 1994; Knapp *et al*, 1985), molecular abnormalities that are related to development and progression of the disorders remain to be clarified.

The $p15^{INK4B}$ gene and its functional homologue $p16^{INK4A}$ gene encode protein negatively regulating the cell cycle by inhibition of cyclin - dependent kinase 4 and 6, which control progression of cells from G1 to S phase (Serrano *et al*, 1993). Inactivation of the $p15^{INK4B}$ and $p16^{INK4A}$ genes by homozygous deletion has been detected in many cancers, suggesting that they are candidate tumor suppressor genes (Kamb *et al*, 1994; Nobori *et al*, 1994). In hematological malignancies, homozygous deletion of $p16^{INK4A}$ / $p15^{IKN4B}$ gene usually occurs in acute lymphoblastic leukaemia (ALL) and non-Hodgkin lymphoma (Herbert *et al*, 1994; Ogawa *et al*, 1995; Tien *et al*, 1998), but rarely in myeloid malignancies (Hirama *et al*, 1995; Uchida *et al*, 1997a). Recently, hypermethylation of 5′- CpG islands within the promoter regions was found to be an alternative mechanism of $p16^{INK4A}$ / $p15^{INK4B}$ gene inactivation (Herman *et al*, 1995; Herman *et al*, 1996a). These GC - rich regions are generally unmethylated in normal tissues, and their methylation is associated with transcriptional silencing of the genes (Merol *et al*, 1995). Hypermethylation of $p15^{INK4B}$ gene, but not $p16^{INK4A}$ gene, was frequently observed in AML and ALL (Batova *et al*, 1997; Iravani *et al*, 1997; Herman *et al*, 1997), suggesting $p15^{INK4B}$ gene inactivation may play an important role in the pathogenesis of these leukaemias. Similar findings were also demonstrated in MDS (Quesnel *et al*, 1998;

Uchida *et al*, 1997b). However the number of reports in literature is limited and the time sequence of the gene alteration is not clear because few patients were serially studied from diagnosis through the course. In this study, methylation status of the $p15^{INK4B}$ promoter region was analyzed on 50 MDS patients, using a methylation specific polymerase chain reaction (PCR) method. Twenty-two patients were serially followed up for two or more times. It was found that the incidence of $p15^{INK4B}$ gene methylation was 16% at diagnosis of MDS, and increased to 34%, usually accompanied with disease progression, during the course. It disappeared after bone marrow transplantation in two patients. $p15^{INK4B}$ gene methylation was highly associated with FAB subtype at study, but not with that at diagnosis. Methylation of $p15^{INK4B}$ detected either at diagnosis or during subsequent follow-up can predict the occurrence of leukaemic transformation.

三、研究方法及步驟

Patients

Methylation status of $p15^{INK4B}$ promoter region was studied on bone marrow cells from 50 patients with MDS. Three of them had a history of aplastic anemia four to ten years before the diagnosis of MDS, while the remaining 47 patients had primary MDS without other underlying hematologic or malignant diseases or exposure to cytotoxic drugs or irradiation previously. The diagnosis and classification of MDS were made according to FAB criteria (Bennett *et al*, 1982). The patients included 33 males and 17 females. Forty-eight patients were adults and two were children with a median age of 53 years (range, 5 to 80 years).

Cytogenetic Study

Chromosome analyses were done as described previously (Tien *et al*, 1994). Bone marrow cells were harvested directly or after 1-3 days of nonstimulated culture. Metaphase chromosomes were banded by the conventional trypsin - Giemsa banding technique and karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN, 1985).

Methylation Specific polymerase chain reaction (PCR)

Analysis of methylation status of the promoter regions of the $p15^{INK4B}$ gene was performed by methylation specific PCR as described (Herman *et al*, 1996b), which is sensitive and specific for methylation of CpG sites in a CpG island. The method consists of two steps: modification of DNA by sodium bisulfite which converts all unmethylated, but not methylated cytosine to uracil, and amplification of the bisulfite-modified DNA by PCR using primers specific for methylated versus unmethylated DNA.

Mononuclear cells were isolated from bone marrow aspirates by Ficoll-Hypaque gradient centrifugation. High-molecular-weight DNAs were extracted as described previously (Tien *et al*, 1994). DNA (4μg) in a volume of 40μl was denatured by addition of 10μl 1M NaOH (final concentration 0.2M) for 10 min at 37℃. Thirty microliter of 10mM hydroquinone (Sigma, St Louis, MO, USA) and 520μl of 1.5M sodium bisulfite (Sigma) at pH5 were added and mixed and samples were incubated under mineral oil at

50℃ for 16hr. Modified DNA was purified using the Wizard DNA purification resin and the Vacuum Manifold according to the manufacturer (Promega, Madison, WI, USA), and then eluted into 100μl of water. Final desulphonation was achieved by treatment with 50μl 1M NaOH (final concentration 0.3M) at room temperature for 5 min, followed by ethanol precipitation. DNA was resuspended in 45μl of water and used immediately or stored at -20% before use.

Bisulfite - modified DNA was amplified by PCR using primer sets designed by Herman et al (1996b) shown as follows: p15-M, 5′-GCGTTCG TATTTTGCGGTT-3′(sense) and 5′-CGTACAATAACCGAACGACCGA-3′(antisense); p15-U, 5′-TGTGATGTGTTTGTATTTTGTGGTT -3′(sense) and 5′-CCATACAATAACCAAACAACCAA-3′ (antisense); p15-W, 5′-CGCACCCTGCGGCCAGA-3′ (sense) and 5′-AGTGGCCGAGCGGCCGG-3′ (antisense). PCR was performed as described previously (Tien *et al*, 1994). Negative controls including normal DNA and distilled water were used in each experiment. To avoid contamination, each DNA sample was aliquoted and analyzed for at least two different PCR.

Statistics

Comparisons were made with χ^2 or Fisher's exact test. Curves of survival were plotted by the Kaplan-Meier method; differences between curves were analyzed by the log - rank test. Multivariate analysis was performed with a Cox regression model. All statistical analyses were done using the SPSS for Windows Release 7.0 (SPSS Inc., Chicago, IL, USA).

四、研究結果

Methylation status of the $p15^{INK4B}$ gene in MDS

Among the 50 MDS patients, methylation status of the promoter regions of the $p15^{INK4B}$ gene was initially studied at diagnosis in 43 patients (11 RA, 2 RARS, 14 RAEB, 12 RAEB-T, and 4 CMML), at transformation to AML in 5 (4 from RAEB & 1, RAEB-T), at the time when RA had progressed to RAEB-T in one, and at 11 months after diagnosis but without progression of the disease in the remaining one (RAEB). The results were summarized in Table 1. Seven (16%) of the 43 patients who were studied at diagnosis showed $p15^{INK4B}$ gene methylation as demonstrated by amplified bands in PCR using p15-M primers which were specific for methylated DNA (Fig.1). The incidence of $p15^{INK4B}$ methylation increased to 24%(12/50) when other 7 patients who was initially studied 3 to 36 months after diagnosis were included in analysis. In addition to the amplified bands shown by PCR using p15-M primers, samples from 9 of the 12 patients with $p15^{INK4B}$ methylation could also be amplified with p15-U primers, which were specific for unmethylated DNA (Fig.1). This might be due to sample contamination by normal cells or presence of unmethylated alleles in the MDS (Quesnel *et al*, 1998) cells. In the remaining 3 patients, only methylated DNA could be detected; 2 of them had RAEBT and 1, AML at study.

Sequential analysis of methylation status of $p15^{INK4B}$ gene in the same patients

Serial analysis of methylation status of $p15^{INK4B}$ gene for two to four times was performed on 22 patients, 2 to 81 months (mean, 19 months) after the first study (Table 2). Five of the 18 patients who did not have $p15^{INK4B}$ methylation of the bone marrow cells at initial study showed positive result subsequently. All these five patients had disease progression to higher-risk MDS (case 7) or to AML (cases 25, 30, 49 and 50) before or at the time of study, compared with five of the 13 patients who remained negative for $p15^{INK4B}$ methylation did so; the difference was statistically significant (p=0.007). All four patients who had $p15^{INK4B}$ methylation at initial study remained so at subsequent study; in two of them (cases 20 & 42), $p15^{INK4B}$ gene converted to unmethylated status after allogeneic bone marrow transplantation. Overall, 17 (34%) of the 50 MDS patients demonstrated methylation of the $p15^{INK4B}$ gene either at diagnosis or during follow-up period (Table 1).

Correlation of methylation status of $p15^{INK4B}$ gene with hematologic features and cytogenetic results

Correlation of methylation status of $p15^{INK4B}$ gene with FAB subtype was summarized in Table 1. The frequency of $p15^{INK4B}$ gene methylation at initial study was significantly correlated with FAB subtype at the time of study (Table 1, 0% in RA & RARS vs 29% in RAEB, RAEB-T & CMML vs 60% in AML evolved from MDS, p=0.019), but not with FAB subtype at diagnosis (p=0.14). This correlation was even more significant if the results of subsequent study were also included for analysis (Table 1, 0% in RA & RARS vs 30% in RAEB, RAEB-T & CMML vs 75% in AML, p=0.001); the same was not true when correlation was made with FAB subtype at diagnosis (p=0.404). The incidence of $p15^{INK4B}$ gene methylation in low-risk (RA & RARS) and high-risk MDS (RAEB, RAEB-T & CMML), respectively, remained stable from diagnosis through the course when FAB subtypes at the time of study were used in analysis: 0% through for the former and from 23% at diagnosis to 30% finally for the latter.

Chromosomal results were available for all patients except the one who had no metaphase cells for analysis. Twenty-five patients showed clonal chromosomal abnormalities, including 18 with single and 7 with multiple aberrations. Monosomy 7 was detected in 9 patients; three of them had additional abnormalities. There was no correlation between $p15^{INK4B}$ methylation at initial study and chromosomal changes: 25% in patients with normal karyotype, 33% in those with monosomy 7, and 19% in those with other abnormalities, p=0.716; 25% in patients with normal karyotype, 28% in those with single abnormalities, and 14% in those with multiple abnormalities, p=0.778. The incidence of $p15^{INK4B}$ methylation in patients with monosomy 7 increased to 56% (5/9) during follow-up period, compared to 33% (12/40) in other patients, but the difference was not statistically significant (p=0.244). Methylation of the $p15^{INK4B}$ gene was also not associated with percentage of bone marrow

blasts at diagnosis.

Correlation of methylation status of the $p15^{INK4B}$ gene with disease progression and survival of the patients

MDS in 18 of the 48 patients who were followed-up for more than 2 months transformed to AML. Methylation status of $p15^{IKN4B}$ gene at diagnosis of MDS could not predict the occurrence of leukaemic transformation: two of the six patients who had $p15^{INK4B}$ gene methylation at diagnosis and been followed-up for more than two months progressed to AML, compared to nine in 35 patients without the gene methylation at diagnosis did so (p=0.651). However, if the results of the study during the course were included, patients with $p15^{INK4B}$ gene methylation at diagnosis or in subsequent study had a significantly higher chance to have disease progression to AML than those without the gene methylation (Table 3, 11/16 or 69% vs 7/32 or 22%, p=0.004). In four patients, methylation of the $p15^{INK4B}$ gene was detected one to six months before leukaemic transformation (two were shown as cases 20 & 24 in Table 2); in six, at the time of evolution to AML; and in one, three months after it (case 30 in Table 2). Of the five patients who had $p15^{INK4B}$ gene methylation but no leukaemic transformation, two received bone marrow transplantation, two died soon after the detection of the gene alteration, and one progressed to higher-risk MDS.

Patients with $p15^{IKN4B}$ methylation at diagnosis had a significantly shorter survival than those without the change (median, 11 mo vs 26 mo, p=0.0303). However, if only the patients with high-risk MDS were counted, there was no survival difference between the two groups of patients with or without $p15^{INK4B}$ methylation (p=0.366). In a Cox multivariate analysis of prognostic implication of FAB subtype, cytogenetics, bone marrow blasts and $p15^{INK4B}$ methylation at diagnosis, which all showed prognostic value for survival by univariate analysis, only FAB subtype and cytogenetics had significant influence on survival time.

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