行政院國家科學委員會專題研究計畫　成果報告

大腸直腸癌之染色體 4，8p，17p，18q 刪除圖譜繪製：腫瘤轉移與預後之分子指標的開發

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Deletion mapping on chromosomes 4, 8p, 17q and 18q in colorectal cancer: Exploration of molecular predictors of tumor metastasis and prognosis

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一、中、英文摘要

大腸直腸癌近年來在臺灣地區十大癌症死因中皆排名第三。雖然目前的研究逐漸了解：多步驟的基因變化在大腸直腸致癌過程中所扮演的角色，但對此癌症的篩檢與預防以及病人治療與預後的評估仍是一個重要的挑戰。已知大腸直腸癌腫瘤中常有多重染色體獲得及漏失(gains and losses of chromosomes)的變化，利用 comparative genomic hybridization 分析得知：在染色體 4, 8p, 17p 和 18q 最常發生染色體漏失的現象，而這些染色體變化與癌症進展、病人存活時間及腫瘤轉移亦有相關，意味著這些染色體區域極有可能存在有與大腸直腸致癌相關的抑癌基因(tumor suppressor genes)，因此，非常值得利用更敏感與明確的方法針對這些常發生染色體漏失的區域進行研究。本計畫針對 106 個大腸直腸癌腫瘤，利用第四條染色體上 22 個微衛星標記(microsatellite markers)以雜接合子漏失 (loss of heterozygosity) 分析方法，研究此染色體上的刪除圖譜(deletion map)。並將以所得之基因變化探討其與病人臨床及病理特徵、腫瘤復發與轉移以及病人存活時間的相關性，以開發大腸直腸癌預後與治療決策的分子指標。

關鍵詞：大腸直腸癌、第四條染色體、微衛星標記、雜接合子漏失分析、刪除圖譜

Abstract

Colorectal cancer (CRC) is the third leading cause of cancer death in Taiwan and remains a significant public health challenge, despite our increased understanding of the genetic mechanisms involved in the initiation and progression of this disorder. Colorectal carcinomas are characterized by the emergence of multiple chromosomal aberrations. By using comparative genomic hybridization (CGH) analysis, losses of chromosomes 4, 8p, 17p and 18q are detected at higher frequency and are associated with stage progression, shorter survival times and tumor metastasis in sporadic cancers. The affected chromosomal regions possibly harbor tumor suppressor genes participating in colorectal carcinogenesis. The conspicuous regions found in the CGH experiments allow the selective and detailed characterization at a molecular level via the detection of loss of heterozygosity. In this project, the deletion mapping on chromosomes 4 in 106 primary CRC were defined by polymerase chain reaction-based allelotyping using 22 polymorphic microsatellite markers. These results will be used to examine the associations of the genetic alterations with clinicopathological variables, tumor recurrence and metastasis, as well as survival of the patients, and to explore the useful molecular predictors for prognosis and stratification in the design of therapy for CRC patients.

keywords：colorectal cancer, chromosomes 4, microsatellite markers loss of heterozygosity, deletion mapping

二、緣由與目的

Colorectal cancer (CRC) is one of the most common malignancies not only in the United States, but also in Taiwan. Despite progress in chemotherapy, radiation therapy, and surgery, there has been little improvement in the survival of patients with CRC during the past several decades. This has prompted scientists to search for clues at the molecular level that may help in understanding colorectal pathogenesis. It has been shown that colorectal tumorigenesis involves multiple steps and the development towards malignancy is associated with the mutation or gain of tumor-promoting oncogenes (e.g. *K-ras*) and the mutation or loss of tumor suppressor genes (TSGs) (e.g. *p53*) [1,2].

Colorectal carcinomas are characterized by frequent recurrent gains and losses of chromosomal material, which may be important in the development and progression of CRC [3,4]. Current models suggest that CRC initiation and progression are secondary to both the activation of oncogenes and the deletion of TSGs. The role of each, however, is still poorly understood, particularly with regard to the induction of metastasis. Recent comparative genomic hybridization (CGH) studies have demonstrated that appreciable numbers of chromosome aberrations are frequent determined in sporadic CRC, most notably loss of chromosome 4, 8p, 17p and 18q [5-8]. These chromosomal alternations are associated with stage progression, shorter survival times and tumor metastasis. Identification of the specific chromosomal regions that are lost in conjunction with the development of invasiveness and tumor metastatic ability, and a search for the TSGs localized to these respective regions, may lead to a more accurate prediction of prognosis.

Because genomic deletion is reflected in a genetic mechanism called loss of heterozygosity (LOH), LOH is common to all human solid tumors and allows the expressivity of recessive loss-of-function mutations in TSGs [9]. Therefore, the detection of recurrent LOH in a chromosomal region is now considered to be critical evidence for the localization of TSGs. On the other hand, LOH are important phenomena in tumor development which have potential diagnostic and prognostic relevance. LOH in the long arm of chromosome 18 can be detected in about 60-70% of CRC cases. The most frequent area of loss is 18q21-qter, where the candidate TSG, *MADR2, DPC4* and *DCC*, are known to reside [10]. LOH in this area had been suggested to be predictive of metastasis and poor prognosis [11-14]. In our previous study, paired normal and tumor samples of 39 patients with CRC were collected and analyzed for replication error (RER) and LOH using a set of 10 microsatellite markers linked to *APC, hMSH2, hMLH1, DCC, P53, NM23, HPC1* and *MET* genes as well as tumor suppressor genes (TSG) on 8p22. RER was observed in 20.5% and had significantly higher frequency in the patients younger than 60 yr (P= 0.049). More than one third of informative tumors showed LOH at *P53, DCC* and *APC* genes. LOH at TP53-Dint marker was significantly associated with survival status (P=0.038) in which a higher frequency was observed in the patients who

died from CRC. About 27% of informative tumors showed LOH at the D8S254 marker that is suspected to be near one or more TSG. The frequencies of LOH at the *NM23*, *hMSH2*, *hMLH1* and *HPC1* genes were 18.5%, 12.1%, 9.1% and 7.4%, respectively [15]. The present results for the sporadic occurrence of CRC in Taiwanese patients further extend the correlation of clinical pathology and prognosis with the analysis of RER and LOH.

三、研究方法

Study population and Tissue Specimens

Both paired normal and tumor specimens were obtained from each of 150 patients with primary colorectal cancer, who underwent surgery between August 1997 and February 2000 at the Departments of Surgery, Cardinal Tien Hospital and Veterans General Hospital-Taipei. For comparing primary loco-regional cancers with liver-metastatic cancers, at least 20 fresh specimens, 10 colorectal adenocarcinomas and 10 liver metastases, were collected. No patients received chemotherapy or radiation therapy prior to undergoing resection of the primary tumor. All specimens were carefully trimmed of all normal adjacent tissues. A frozen section of the specimens was done to confirm the histological diagnosis and the absence of normal adjacent contaminating tissue. The samples were frozen immediately following resection and stored at –80℃ until DNA extraction.

DNA Preparation

High-molecular-weight DNA was prepared from each tumor and corresponding normal tissue using QIAamp Tissue Kit (QIAGEN GmbH, Germany). The tissue was cut into small pieces 25mg each, mixed with 180 μL of Buffer ATL and 20 μL of Proteinase K (15mg/mL), and then incubated at 55℃ until the tissue was completely lysed. The lysate was mixed with 200 μL of Buffer AL, incubated at 70℃ for 10 min, and followed by adding 210 μL of ethanol (96-100%). In the final step, the DNA was collected in a QIAamp spin column, washed with Buffer AW, and the DNA eluted with Buffer AE.

Fluorescent PCR Amplification of Microsatellites

Normal and tumor genomic DNA sample pairs were amplified using fluorescence-labeled primers retrieved from Applied Biosystems PRISM Linkage Mapping Set-MD10 (Applied Biosystems, CA., USA), comprising 22 polymorphic microsatellite markers located on chromosomes 4. These markers are with an average spacing of 9.2 cM and average heterozygosity of 0.79. In each of primer pair, the 5' primer was synthesized with FAM, TET or HEX fluorescent label. Amplifications were done in a final volume of 20 μL using 10 nM of each of the respective primers, 25 ng of normal or tumor DNA, 125 μM of each dNTP, 1 unit AmpliTaq Gold DNA Polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl,

and 2 mM $MgCl_2$. PCR was carried out in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems), as follows: a 10 min pre-PCR incubation step at 95℃; 30 cycles of 96 ℃ for 10 sec, 55℃ for 30 sec, and 70℃ for 3 min; and a final extension of 70℃ for 30 min.

Detection and Analysis of Microsatellites

The PCR products were electrophoresed on an ABI 377 automated sequencer (Applied Biosystems). Normal and tumor DNA pairs were compared for the changes in the number of allele peaks scored and peak height of each fluorescent marker via GeneScan and Genotyper software (Applied Biosystems).

Definition of Allelic Loss (LOH)

For a given informative marker, an LOH index of ≦0.67 or ≧1.5 indicates allele loss. This equates to a 33% decrease in peak height of one of the tumor alleles as compared to the normal allele.

四、結果與討論

One hundred and six tumor samples from patients with CRC were analyzed for loss of heterozygosity on chromosome 4. Twenty two polymorphic microsatellite markers located on this chromosome were used in the allelotyping study. Analysis of 22 microsatellite markers identified 62 samples (58.5%) exhibiting loss of heterozygosity of chromosome 4 in at least 1 locus. Eight tumors revealed loss at all informative markers, indicating the probable presence of monosomy. Fifty four tumors displayed partial loss of heterozygosity and delineated 7 frequently deleted regions (2 on 4p and 5 on 4q). These were the (1) D4S2935 locus, (2) D4S391 locus, (3) D4S2964 locus, (4) D4S414 locus, (5) D4S402 locus, (6) D4S413 locus, and (7) D4S426 locus. Of these 7 loci, loss of heterozygosity at D4S2964, D4S402 and D4S413 loci occurred in more than 32% of the tumors. Twenty seven (25.5%) of the 106 tumors exhibited microsatellite instability in at least one of the tested microsatellite markers. Microsatellite instability occurred in 2.1% (49 of 2329) of the loci tested and the percentage of altered loci in the 27 tumors ranged from 4.6-45.5%. These results demonstrate a high frequency of chromosome 4 loss in CRC and might demarcate 7 putative tumor suppressor loci involved in the development and progression of CRC.

五、計畫成果自評

We have defined 7 frequently deleted regions on chromosome 4 in CRC. These results suggest that there may be multiple putative tumor suppressor genes located on both arms of

chromosome 4 whose inactivation are important in colorectal carcinogenesis. Therefore, it is worthy to precisely map tumor suppressor loci within these 7 regions for further positional cloning efforts.

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