計畫成果報告

計畫名稱: 利用雷射顯微分離系統來分析肝癌的基因變化

Molecular Genetic Analysis of Hepatocellular Carcinoma by Laser Capture Microdissection

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中文摘要

關鍵字: 肝細胞癌、雷射顯微分離系統、抑癌基因

肝細胞癌是全世界最常見的癌症之一，也是國人癌症死因的第一位。其發生的主要原因與B型肝炎病毒的慢性感染有關。此外C型肝炎病毒也可能有關。其他可能的因素包括黃麴毒素的污染，然而真正的分子致病機轉目前尚未清楚。近年來癌症的研究趨向於有系統的分析比較正常細胞及癌細胞間 DNA 或 RNA 的差異。使用的方法包括區分顯示法(DD)，基因表現連序續分析法(SAGE)，微晶片雜交系統(gene chip hybridization system)等等。然而這些高度精密的方法確受限於檢體的 DNA，RNA 不是均質的(homogeneous)，所得的結果並不十分理想。

一般 solid tumor 在撿取腫瘤細胞時容易污染其它正常細胞，造成分析不夠精確。而雷射顯微分離系統(laser capture microdissection, LCM)，它可以精確快速、自動化的取樣，避免交叉污染，而且不像傳統的 microdissection 那樣耗費時間及人力。在顯微鏡下將這些癌組織有興趣的部份，如癌前細胞(precancerous cells)，癌細胞(cancer cells)或是具侵襲性的癌細胞(invading groups of cancer cells)，精確的利用雷射顯微分離系統取得，然後萃取其 DNA 或 RNA，直接分析 DNA 或建立 cDNA library 比較癌前細胞(precancerous cells)，癌細胞或是具侵襲性的癌細胞(invading groups of cancer cells)之間的基因差異。美國國家癌症基因大計劃即利用此系統來研究美國常見的癌症(包括肺癌、攝護腺癌、乳癌等)，目前已有不錯的結果。

在先前的研究中，我們利用比較性雜交法(comparative genomic hybridization)及微小衛星分析法(microsatellite analysis)分析肝癌的病人，結果顯示發生基因丟失最高的染色體是 16q，且與家族性的肝癌病人有統計上的意義。因此，在本研究中，我們選取 30 例肝癌組織，以雷射顯微分離系統(laser capture microdissection, LCM)取得檢體，然後萃取其 DNA，利用位於 16q 的微小衛星來做進一步的細微定位，尋找可能與肝癌有關的抑癌基因或是與癌症轉移有關的基因。LOH 頻率較高的 primer 包括 D16S409, D16S419, D16S415, D16S390, D16S3034，且細微定位的結果靠近 16q12.1，再次印證先前的結果。LOH 頻率高於先前未使用 LCM 所製備的檢體，由此可知 LCM 確實可以避免交叉污染，使所得的結果更正確。本計畫成功地建立 LCM 系統，包括 DNA 或 RNA 的萃取，將來可以利用 LCM 系統所得的檢體做更進一步的分析。

Abstract

Keywords：Hepatocellular carcinoma (HCC), laser capture microdissection(LCM), genetic profile

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and is the leading cause of cancer mortality. Nevertheless, the molecular mechanism remains to be clarified. Cancers arises from the accumulation of genetic changes. Identifying the genetic changes in the tumor cells seems a good method by directly comparing the genes of tumors with the non-tumor parts. The value of even the most sophisticated genetic testing methods will be limited if the input DNA, RNA, or proteins are not derived from pure populations or are contaminated by the wrong cells.

Recently, a laser capture microdissection(LCM) system, developed by NIH(USA) and Arcturus Inc., have overcome the drawbacks of current tissue microdissection techniques. Under the microscope, the diseased cells of interest, such

as precancerous cells or invading groups of cancer cells, are surrounded by these heterogeneous tissue elements. We can get pure cells from specific microscopic regions of tissue sections by LCM system. Therefore, microdissection is essential to apply molecular analysis methods to study changes in actual tissue. The LCM system has been applied to study the genetic profiles between cancer cells and normal cells in the Cancer Genome Anatomy Project (CGAP).

In our previous study, we found that HCC patients tend to have higher frequency of gene loss at chromosomal arm 16q. Since 16q is important, we plan to select microsatellite markers in 16q to identify minimal deleted regions. In this project, we analyzed the genetic profile of 30 HCC patients by using LCM method, microsatellite analysis. The highest percentage of LOH was shown for loci at D16S409, D16S419, D16S415, D16S390, D16S3034. The frequency of LOH seemed to have higher LOH frequency than previous study without LCM preparation. We also confirmed the most frequent regions of LOH for HCC are 16q12.1

Based on this study, Analysis of pure cell population and read its molecular changes will enable us to identify precisely what is different between normal cell and cancer cell. We hope that through this study we can have a better understanding of the molecular mechanism of hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and is the leading cause of cancer mortality. Chronic hepatitis B viral(HBV) and hepatitis C viral (HCV) infection are regarded as important factors in the development of HCC. Nevertheless, the molecular mechanism remains to be clarified.

Cancers arises from the accumulation of genetic changes. Identifying the genetic changes in the tumor cells seems a good method by directly comparing the genes of tumors with the non-tumor parts. With the advent of polymerase chain reaction (PCR) and the development of high throughput, automated micro-hybridization arrays and mutation screening methods, DNA or RNA can be extracted from tissue biopsies and analyzed with a parallel panel of hundreds or even thousands of genetics markers. The value of even the most sophisticated genetic testing methods will be limited if the input DNA, RNA, or proteins are not derived from pure populations or are contaminated by the wrong cells.

Recently, a laser capture microdissection(LCM) system, developed by NIH(USA) and Arcturus Inc., have overcome the drawbacks of current tissue microdissection techniques. It is simple, requires no moving parts, involves no manual microdissection or manipulations and enables one-step transfers. The use of sterile, disposable transfer films minimizes potential contamination, which is particularly important for PCR-based analyses. Under the microscope, the diseased cells of interest, such as precancerous cells or invading groups of cancer cells, are surrounded by these heterogeneous tissue elements. We can get pure cells from specific microscopic regions of tissue sections by LCM system. Therefore, microdissection is essential to apply molecular analysis methods to study changes in actual tissue. The genetic profile of specific cell populations or molecular differences between normal cells and tumor cells can be analyzed. The LCM system has been applied to study the genetic profiles between cancer cells and normal cells in the Cancer Genome Anatomy Project (CGAP).

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study, Analysis of pure cell population and read its molecular changes will enable us to identify precisely what is different between normal cell and cancer cell. We hope that through this study we can have a better understanding of the molecular mechanism of hepatocarcinogenesis.

Methods

Microdissection:

Unstained thin(about 12 μm) frozen tissue sections will dissected under microscopic visualization. A pure population of liver cells will be dissected. An adjacent H&E-stained section will be used as a guide to ensure accuracy of dissection.

A laser beam and special transfer film are used to lift the desired cells out of the tissue section, leaving all of the contaminating or unwanted cells behind. The transparent transfer film is applied to the surface of the tissue section. Under the microscope, the diagnostic pathologist or researcher views the tissue through the film and chooses microscopic clusters of cells to study. When the cells of choice are in the center of the field of view, the operator pushes a button which activates a laser diode integral with the microscope optics. The pulsed laser beam activates a precise spot on the transfer film immediately above the cells of interest. At this precise location the film melts and fuses with the underlying cells of choice. When the film is removed, the chosen cell(s) remain stuck to its undersurface, while the rest of the tissue is left behind.

DNA and RNA extraction:

Genomic DNAs will be extracted from the tumor and non-tumor liver tissues using classic method as described before[5].

RNA will be extracted from the tumor and non-tumor liver tissues using the single step extraction method with guanidinium thiocyanate-phenol-chloroform.

Microsatellite Polymorphism Analysis

1. All the HCC samples including tumor and non-tumor part will be systematically studied by using the microsatellite markers by using primers in chromosome 16q.
2. The primers specific for these markers are commercially available(Research Genetics, Huntsville, AL)(6). Each marker will be amplified by the polymerase chain reaction(PCR) in 25 μl volumes of a mixture containing 25 ng of genomic DNA template, 5 pmol of each primer, 75μM of each deoxyguanosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate, 7.5μM triphosphate deoxyadenosine triphosphate, 1.5 mM magnesium, 1X PCR buffer, 1.25μCi of [^{33}p]dATP 5U/μl, and 0.5U *Taq*. The PCR will be performed in a thermocycler which can fit 96 microtiter plate (PTC-100-96V, MJ Research Inc., Watertown, MA). Reactions will be performed in 27 cycles under the following conditions: 30s at 94^{0}C for denaturation, 75s at 55^{0}C for primer annealing and 30s at 72^{0}C for primers extension. Finally PCR product will be further incubated in 72^{0}C for another 6 minutes.
3. After completion of PCR, the PCR products will be run on a 6% PAGE gel followed by exposure of the gel to X ray film for 1 to 2 days. The band pattern between tumor and non-tumor part will be compared. The genetic changes including the LOH and microsatellite instability will be analyzed.

Results

DNA and RNA preparation from LCM

In this study we have succeeded to prepare the DNA or RNA (Figure1, 2) from

microdissected tissues from OCT slide.

LOH and fine mapping analysis of 16q in HCC

In this study we use microsatellite marker to analyze 30 cases of HCCs, most of them are small in size, in order to study the genetic changes of HCC and to further narrow down the common LOH sites in 16q.

A total of 5 microsatellite markers in chromosome 16q were used to study the LOH of HCC. The highest percentage of LOH was shown for loci at, D16S409, D16S419, D16S415, D16S390, D16S3034.(Table1). The minimal deleted region was mapped to 16q12.1, and the LOH frequency was higher than previous data without LCM preparation.

Discussion

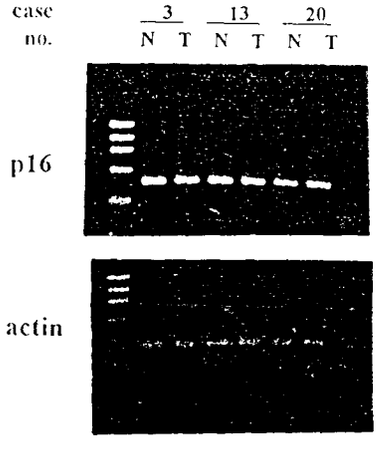
Our preliminary result shows that HCCs in Taiwan have frequent LOH in chromosome 16q. In this study, HCC seems to have common LOH sites at 16q12.1 by LCM and microsatellite analysis. These data suggested that the putative tumor suppressor genes might lie in these regions.

In the future, we will apply the LCM system and microarray system to assay HCC samples. Analysis of pure cell population and read its molecular changes will enable us to identify precisely what is different between normal cell and cancer cell.

Table 1 Loss of heterozygosity at 5 microsatellite loci on chromosome 16q in HCCs

Locus Symbol	*Chromosomal location*	*(LOH informative cases)*	*Frequency of LOH(%)*
D16S409	16q12.1	7/15	47
D16S419	16q12.1	9/20	45
D16S415	16q12.1-q13	11/17	65
D16S390	16q12.1	11/22	50
D16S3034	16q12.1-q13	3/8	38

圖 ：以反轉錄-聚合酶連鎖反應分析 p16 基因 mRNA 表現的情形



圖二：染色體 16q 上微小衛星標記雜合子丟失的例子