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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遺傳性大腸癌的基因分析

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計畫主持人：魏淑鉁

共同主持人：翁昭旼, 吳忠勳

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Preparation of NSC Project Reports

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主持人：魏淑鈐　台大醫學院 內科

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Abbreviated title: **mutation screening of MSH2 and MLH1 in HNPCC**

INTRODUCTION

Colorectal cancer (CRC) poses a major public health problem in most Western nations, and it also becomes an increasing leading cause of death due to cancer in Eastern countries. In Taiwan, the prevalence rate of colon cancer increased in recent years gradually and it becomes the third leading causes of death due to cancer in recent years, according to the official reports from the department of health, government of Taiwan. It was predicted that at least 10% of the CRC burden be attributed to a primary genetic factor (Lynch, Smyrk, 1999). And the most common hereditary CRC disorder is hereditary nonpolyposis colorectal cancer (HNPCC) (Mecklin et al., 1994).

The diagnosis of HNPCC is based on the so-called "Amsterdam criteria", which require (1) three or more relatives with histological verified CRC, one of whom is a first-degree relative of the other two; (2) CRC involving at least two generations; and (3) one or more CRC cases diagnosed before the age of 50 years (Vasen et al., 1991). Several studies for this disease have shown that HNPCC to be a simple Mendelian disease involving five DNA mismatch repair (MMR) genes, *MSH2, MLH1, PMS1, PMS2* and *MSH6* (also called as GT binding protein, *GTBP*) (Akiyama et al., 1997; Bronner et al., 1994; Fishel et al., 1993; Leach et al., 1993; Lindblom et al., 1993; Mikaki et al., 1997; Nicolaides et al., 1994; Papadopoulos et al., 1994; Peltomaki et al., 1993).

Published studies suggest that the majority of DNA MMR gene mutations associated with HNPCC occur in the *MSH2*and *MLH1* genes (Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Nicolaides et al., 1994; Palombo et al., 1995; Liu et al., 1994; Wijnen et al., 1995; Tannergard et al., 1995; Hamilton et al., 1995; Nystrom –Lahti et al., 1996; Liu et al., 1996; Kohonen-Corish et al., 1996; Kolodner et al., 1995; Mauillon et al., 1996; Viel et al., 1997; Luce et al., 1995; Han et al., 1995; Moisio et al., 1996; Nytrom-Lahti et al.,1995; Ionov et al., 1993). 60% of HNPCC kindreds could be associated with mutations in *MSH2*, and 30% could be associated with *MLH1* (Fishel et al., 1994, Nystrom-Lathi et al., 1994; Kolodner et al., 1995). The incidence of MMR gene mutations in HNPCC kindreds reported in previous series varies considerably, ranging from 22% to 86% (Wijnen et al., 1995; Nystrom-Lahti et al., 1996). Identifying mutations in HNPCC kindreds allows identification of mutation carriers that would benefit from screening and surveillance. In order to set-up the screen system and to realize the genetic background of HNPCC kindreds in Taiwan, this study was aimed to identify germline mutations of the two major HNPCC-predisposing genes.

MATERIALS AND METHODS

Patients

This study involved 10 families from the National Taiwan University Hospital. All of them fulfill the Amsterdam criteria. Initial screening was performed on a single proband from each of the 10 unrelated families. Whenever they were available, additional family members were tested for the segregation of specific molecular variants. A 20-ml blood sample was drawn in EDTA from each individual tested after genetic counseling and obtaining the consent.

RT-PCR

Lymphocytes were isolated from 20ml of whole blood by using the Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB SE-751 84 Uppsala Sweden) and centrifugation. Total RNA isolation was performed by using the RNeasy kit (Qiagen), according to the manufactures. Two micrograms of total RNA was reverse transcribed using 1μg of random hexamers, 4 mmol/L of deoxynucleoside triphosphates, and 300 U of Superscript II reverse transcriptase (Life Technologies, Arlinton Heights, IL) using the manufacture's suggested reaction conditions. Polymerase chain reaction (PCR) amplifications were performed in 25 μL reactions containing the following: 1-2 μL of the complementary DNA mix, 2.5μL of 10X PCR buffer, 0.8mmol/L deoxynuceoside triphosphates, 5 U OptiPol DNA polymerase (GeneTeks, BioScience Inc.), and 5 pmol of each PCR primers. Primers for both *hMSH2* and *hMLH1* were designed to amplify the genes into three overlapping segments about 1 kilo-base in each segment (Figure 1). The upstream primer of each segment contained signals for efficient transcription and eukaryocytic translation (T7 promoter sequence and a translation initiation site). The primers used for the PCR were shown in Table 1. The reactions were programmed for thermal cycling as follows: 35 cycles of 95°C for 30 seconds, 58 to 62°C for 30 seconds, and 70°C for 2 minutes, and a final extension at 70°C for 7 minutes. The PCR products were purified by using the PCR clean up purification kit (Viogene, Sunnyvale, CA 94086), which were then ready for in vitro transcription/translation and automatic sequencing reaction.

IVTT (In vitro transcription and translation)

Purified RT-PCR DNA was mixed with T7 RNA polymerase and 1μ Ci ^{35}S-L-methionine (translation grade, 1000 Ci/mmol at 10 mCi/mol; NEN™ Life Science Products, Inc. Boston, MA 02118) in a rabbit reticulocyte lysate based transcription/translation system (TNT, Promega) and incubated at 30°C for two hours. Protein electrophoresis was performed in 4% stacking/ 12 % separating SDS-polyacrylamide gels, the gels fixed, dried under vacuum at 80°C, and then exposed to X-Omat AR film (Kodak) for 6-24 hours.

DNA Sequencing

Sequencing reaction was performed using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems. Inc., CA). Electrophoresis was carried out using a Genetic Analyzer 310 (PE Applied Biosystems, Inc., CA) equipped with long-read sequencing capillary and POP-6 sequencing polymer (PE Applied Biosystems, Inc., CA). Primers used for direct sequencing reactions are shown in Table 2.

Results

A total of ten families with 202 persons, 52 registered affected individuals, who met the Amsterdam criteria were included in this study. There were 52 persons in these 10 families who had the diagnosis of cancers, twenty-six men and twenty-six women. Six of them had two types of cancer. Most of them had colorectal cancer (40/52), and other kinds of cancers included the endometrial (5/52), ovarian (2/52), gastric (5/52), pancreatic (1/52), lung cancer (1/52),

breast cancer (1/52), thyroid cancer (1/52) and liver cancer (2/52). Among the 52 persons with cancers, the age of cancer onset could be traced in 27 persons. The age at cancer diagnosis ranged from 19 to 64 year-old, with a mean of 42.1 year-old (complete data submitted to J Formosa Med Association).

After completing the studies as mentioned above, we only find one *hMLH*1 mutation in these ten families. The mutation was resulted from deletion of exon 16 of *hMLH*1, which made the expected protein decreased 8kb in size. A shorter PCR fragment was consistently in three of the affected individuals in the same family, examined by using different primer combinations covering the exon 16. The PCR results are shown in Fig.1 and the IVTT results in Fig.2. The sequencing result is shown as Fig.3. However, no other mutation could be found in the other nine families.

Discussion

According to the previous reports, a majority of the germline mutations in the MMR genes in HNPCC patients were affected either MSH2 or MLH1 and were quite unevenly distributed, with some clustering in MSH2 exon 12 and MLH1 exon 16 [Pelmomaki P, Vasen HF, 1997; Wijnen et al., 1996]. Our finding that exon 16 deletion in MLH1 related to HNPCC has not been reported in other series previously. Palmirotta et al. had reported that the transcripts with splicings of exons 15 and 16 of the hMLH1 gene appeared in normal lymphocytes (Palmirotta et al., 1998). However, we have checked our findings to the affected and non-affected persons in the same family and also other families without the mutation, and we only found the affected persons to have the mutation. We believe this mutation has the pathogenic meaning.

Although the clinical pictures (age of cancer onset, right side colon predominant, cancer types) in our series were similar to the previous report (Lynch HT&Lynch JF 2000) (data submitted to the J of Formosa Med Association already), the results of genetic analysis seemed to be quite different. The mutation rate of the hMSH2 and hMLH1 was lower than the previous reports (Wijnen et al., 1995; Nystrom-Lahti et al., 1996). The possible explanations might be related to sampling methods, methods used to detect mutations, number of families analyzed and differences come from ethnology.

Many of the previous reports in estimating the mutation rates of hMSH2 and hMLH1 had included part of results from non-Amsterdam's criteria or modified criteria (Bai et al., 1999; Liu et al., 1998; Nomura et al., 2000; Wang et al., 1999; Weber et al., 1999). This might increase or decrease the mutation rate. And prior selection factors that include screening proband tumors for the microsatellite phenomenon (Liu et al., 1996), linkage and/or mutation analysis (Liu et al., 1994; Wijnen et al., 1995; Wijnen et al., 1996, Liu et al., 1996) are also possible bias to affect the estimated mutation rate. Our study was based on only clinical history, and all families were compatible with the Amsterdam's criteria.

Methods have been used to detect the mutation in MSH2 and MLH1 genes included DGGE (degenerative gradient gel electrophoresis), SSCP (single strand conformation polymorphism), IVTT (in vitro transcription translation), HA (heteroduplex analysis) and direct sequencing (Fidalgo P et al., 2000; Froggatt NJ et al., 1996; Liu T et al., 1998; Weber et al., 1997). Based on the studying materials, they can be divided into RNA-based and genomic DNA-based analyses. Optimal mutation detection methods remain controversial (Kohonen-Corish et al., 1996). Several authors have raised concerns about RNA-based MMR-gene analysis, citing significant numbers of false-positive results (Xia et al., 1996). Kohonen-Corish et al. concluded to confirm the results in at least two patients per family or from genomic DNA. In our series, we had confirmed the results in three patients in the same family. We also checked our methods by detecting the mutations in colon cancer cell lines. The results showed that the methods we used are reliable to detect the mutations in LoVo and

HCT 116.

In considering the effect of sample size to the mutation rate, the sample size in previous literatures ranged from 14 to 75 kindreds (Nicholas et al.,1997; Wang et al., 1999), mostly from 14 to 20. Therefore, we do not think the sample size will affect the mutation rate so much. But we still keep on searching new HNPCC families in Taiwan to get more larger sample size.

Finally, the difference comes from the ethnology. The mutation rate varied from countries to countries, the difference among ethnology is without doubt. But in the report from Weber et al., and Lewis et al.,(Weber et al., 1997; Lewis et al., 1996), they also found lower rate of MSH2 and MLH1 mutation. They both thought that there might be other genes causing the HNPCC, the genetic base still need to be explored. In our opinion, we will check the other three MMR genes (PMS1, PMS2, GTBP) at first. And we will also check the expression level of MSH2 and MLH1 genes to further confirm our results.

In summary, after completing the RNA based genetic scanning for hMSH2 and hMLH1 of 10 HNPCC kindreds in Taiwan, we only found 10 % mutation rate (one MLH1 mutation-exon 16 deletion). Possibilities to this result had been discussed and further studies are already going on to further confirm this result.

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計畫成果自評：

因時間與經費有限（實驗用只核發約30萬5仟元），本計畫本來預計要完成HNPCC及FAP的基因分析，目前只完成了HNPCC部分，但預計仍可發表二篇論文，關於FAP部分，本人希望再申請其他補助，以完成研究。

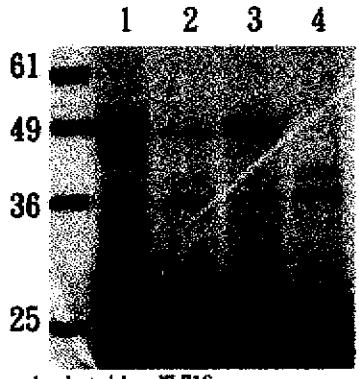
Table 1.

Genes	Names	Primer sequences
hMSH2		
PCR primers	T7-1	5'GGATCCTAATACGACTCACTATAGGGAGACCACCATGCAGCCGAAGGAGACGC3'
	SP6-1	3'TAGGTGACCAATTGTTCAGAA5'
	T7-2	5'GGATCCTAATACGACTCACTATAGGGAGACCACCATGGGACAGTTTGAACTGACTAC3'
	SP6-2	3'CTGTGCACTCGTTTCGACTG5'
	T7-3	5'GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAAGCCCAGGATGCCATTG3'
	SP6-3	3'CGAATAGTTATAATGGAAGTAAGGTAATGACCC5'
hMLH1		
PCR primers	T7-1	5'TAATACGACTCACTATAGGGAGACCACCATGCGGCGGCTGGACGAGACAGT3'
	SP6-1	3'GACTCCATGTCCTTACCCACAC5'
	T7-2	5'TAATACGACTCACTATAGGGAGACCACCATGAACTACTCAGTGAAGAAGTGC3'
	SP6-2	3'CTACGACACGGTTCCGGGTG5'
	T7-3	5'TAATACGACTCACTATAGGGAGACCACCATGTGTACCCCCCGGAGAAGGAT3'
	SP6-3	3'CTAGTCCGTCCAATCGTTCG5'

Table 2.

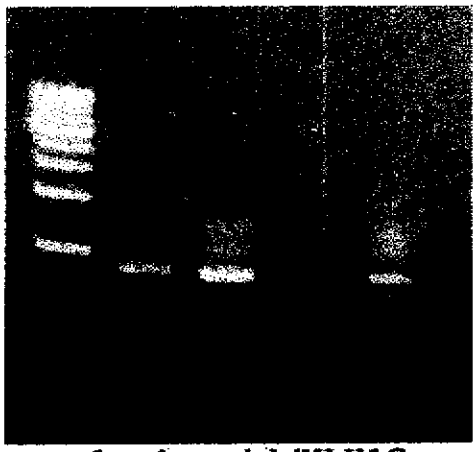
Genes	Names	Primer sequences
hMSH2		
Sequencing primers	SP1	TTGGAGAGCGCGGCCGA
	SP4	CAGGTTGGAGTTGGG
	SP7	CAGAGCCCTTAACC
	SP10	GATCCTAATCTCATCAGTG
	SP12	GCTCAGCTAGATGCT
	SP15	CTGCCTTGGCCAATCAG
hMLH1		
Sequencing primers	SP1	TGAACCGCATCGCGGCGG
	SP4	GGAAGTTGTTGGCAG
	SP7	TTCCTCCAGGATGT
	SP9	CAAAGGGGACTTCAG
	SP10	GAGTCTCCAGGAAG
	SP12	CTGAAGAAGAAGGCTG

Fig. 1



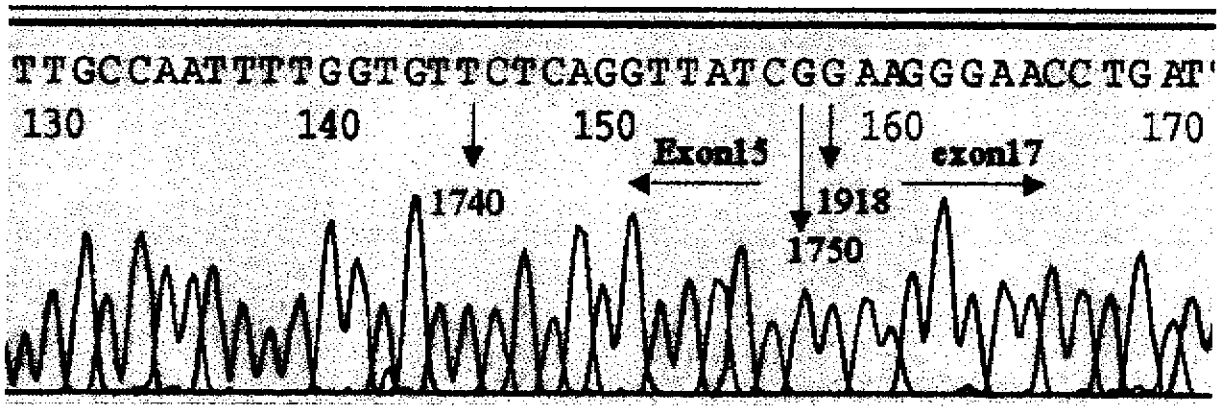
1:plasmid MLH1C
2:k6 MLH1C
3:k6 MLH1C-0.8K
4:k6 MLH1C-0.6K

Fig.2



1. plasmid/MLH1C
2. CONTROL/MLH1C
3. K3/MLH1C
4. K6/MLH1C

Fig. 3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140
150
160
170
Exon15
exon17
1740
1918
1750