行政院國家科學委員會補助專題研究計畫成果報告

幽門桿菌與胃癌有關之致病因子研究

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中文摘要

關鍵字：幽門桿菌，胃癌，致病因子

胃癌是台灣一個重要的疾病，雖然近年來稍有下降，但仍居前五位癌症死亡原因之一。幽門桿菌於 1982 年自人體分離成功後，許多流行病學研究顯示感染此菌與胃癌發生有密切關係，近年日本學者成功的在動物模式以幽門桿菌感染引發胃癌更加強了二者間的關連。但感染此菌人數眾多，卻只有部份人發生胃癌，這些差異可能在宿主遺傳背景,環境因子或菌種之致病因子。若能發現與胃癌有關的特殊致病因子將大有助於早期診斷,與了解致命機轉。國外學著曾報告 CagA，VacA 是可能的致病因子，但在東方的研究卻不然，我們曾初步以血清篩檢與分離出一菌株蛋白，可能與胃癌有關，但因其敏感性不足，本計劃擬利用更系統化的菌斑(Plaque)篩檢整段幽門桿菌基因體,包括用基因比較不同的蛋白表現及 RNA 表現，有差異的基因將分別選殖出來以基因工程表現探討其功能,並常試做早期診斷的標誌。另在幽門桿菌分別將基因破壞，比較原型(wild type)和變異株在體外對胃細胞株或動物模式感染致病力的差別。

本計畫中發現此 26 kD 蛋白質抗體陽性病人有較高的機會發生胃癌，但因病例數少尚未達統計上有意義之差別。另外以微陣列發現有 11 個基因在胃癌病人分離菌株表現較高。

英文摘要

keyword: *Helicobacter pylori*, gastric cancer, virulence factor

Helicobacter pylori was initially isolated in 1982 from the gastric mucosa of a patient with gastritis and peptic ulceration. Since then, increasing evidence suggests that *H. pylori* profoundly influences duodenal ulcerogenesis. Other investigations have demonstrated that *H. pylori* infection is associated with adenocarcinoma and low grade lymphoma of the stomach (MALToma). However, despite the high prevalence of this infection, only a small portion of infected patients incur significant clinical sequelae. Most patients remain asymptomatic throughout their lives.

The infection can not be eradicated in all patients owing to the related cost and possible drug resistance. The difference in clinical outcomes may be ascribed to the host genetic background, environmental factors and virulence of bacterial strains.

We have found a species specific protein in *Helicobacter pylori*, however, because this antigen seems to have limited sensitivity. We try to explore further candidate virulence factors (or marker) associated with gastric cancer by systemic plaque screening of RNA and protein expression and detail functional characterization will be done in the 26kD antigen as well as novel virulence factors. In addition, an animal model of *Helicobacter pylori* induced gastric cancer has recently been established [17,18], we will adopted this model for study of these novel virulence factors.

Our results revealed 11 genetic loci expressed significantly higher in strains from patient with gastric cancer. Nested control study suggested a higher risk in patents positive for antibody to the 26kD protein, however, it was not statically significant due to limited case number.

Helicobacter pylori, a spiral gram-negative bacteria, was initially isolated in 1982 from the gastric mucosa of a patient with gastritis and peptic ulceration. Since then, increasing evidence suggests that *H. pylori* profoundly influences duodenal ulcerogenesis [1]. After *H. pylori* eradication, the relapse rate of both duodenal and gastric ulcers dramatically decreases; a cure for this chronic relapsing

disease has been documented in different areas [2-4]. Other investigations have demonstrated that *H. pylori* infection is associated with adenocarcinoma and low grade lymphoma of the stomach (MALToma) [5-9]. However, despite the high prevalence of this infection, only a small portion of infected patients incur significant clinical sequelae. Most patients remain asymptomatic throughout their lives.

The infection can not be eradicated in all patients owing to the related cost and possible drug resistance. The difference in clinical outcomes may be ascribed to the host genetic background, environmental factors and virulence of bacterial strains. Finding a candidate marker to differentiate the strains that are more harmful to the host would be useful in selecting patients for eradication treatment. According to previous studies, the presence of cagA and the variation of signal sequence in vacA are significantly related to its clinical outcome in western countries [10-13]. However, no such genetic difference apparently exists in most strains in Asia [14-16].

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Bacteria Strains

Biopsy specimens from the gastric body and antrum were taken from patients with gastritis, peptic ulcer disease, MALT lymphoma and adenocarcinoma of the stomach for bacterial culture. Bacteria were grown under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37℃ in Brucella broth containing 5% fetal calf serum. The strains were stored at –80℃ after successful culture and drug sensitivity tests. Strains obtained from American Type Culture Collection (ATCC) and a Canadian strain, UA802 (gift from Dr. DE Taylor, University of Alberta, Edmonton, Canada), were used for comparison. The minimal inhibitory concentrations (MICs) of antibiotics in *E. coli* and *H. pylori* were determined by E-test and Agar dilution methods.

Construction of Expression Libraries

Chromosomal DNA from the interested *H. pylori* strain will be partially digested with the restriction endonuclease Sau3AI. DNA fragments 1 to12 kb in size were harvested from 0.8% agarose gels with the GenecleanIII kit (Bio 101, La Jolla, CA, USA) and ligated into a BamHI-digested and alkaline-phosphatase-treated expression vector, λ -ZAPII. The recombinant λ -ZAPII plasmids will be packaged into lambda phage particles and used to infect *E. coli* XL1-Blue MRF'. A filamentous helper phage was then added to excise the pBK-CMV phagemid in *E. Coli* XLOLR.

Northern screening

(1)Total RNA Isolation. Bacterial cells were grown to mid-log phase on Columbia agar plates, collected, washed with TE buffer (PH=7.4) and pelleted. Cells pellets then will be resuspended and lysed in boiled 1%SDS-TES buffer (50mM Tris-hydrochloride (pH=8.0), 1mM EDTA, and 50mM NaCl) for 5 minutes, proceed with several saturated phenol then extracted twice with phenol:chloroform:isoamyl-alcohol solution, precipitated with equal volume of isopropanol, stored at -70 ℃ or resuspended in DEPC treated water until use.

(2)mRNA Enrichment. Purified RNA will be digested with RNase-free DNase I (Boehringer Mannheim). Subtraction of rRNA was performed according to a previously described method (Plum and Clark-Curtiss, 1994) [17]. *H. pylori* 23S and 16S gene fragments will be amplified by PCR and the primers will be designed according to the published

full genome sequences. The products were cloned separately into *pCR2.1* and *pCRII* vector (Invitrogen) [18]. The recombinant plasmids then were used as templates to generate antisense 23S and 16S RNA *in vitro* with RNA transcription kit (Boehringer Mannheim) using vector bacteriophage T7 promoter and incorporating DIG-modified UTP during transcription reaction. To enrich the mRNA, a twofold excess of DIG-modified antisense rRNA was combined with total RNAs in a volume of 50 μ l of hybridization buffer. This mixture will be overlaid with 50 μ l of mineral oil, incubated at 70℃ for 10 min to denature secondary structures and then was hybridized overnight at 55℃. The mixture will be then incubated with 100 μ l of a suspension of anti-DIG paramagnetic beads (Boehringer Mannheim) for 30 minutes at 25℃. Beads were extracted with magnetic particle separator (Boehringer Mannheim), thereby removing DIG-labeled rRNA hybridized complexes, yielding a fraction enriched for mRNA.

(3)Northern Blots. The mRNA are enriched will be labeled by fluorescence or DIG as probes. The library was plated at a density of 2 x 104 plaques on 150-mm-diameter petri dishes. Plaques were then transferred to two membranes in duplicate onto Nylon membrane (MSI) by vacuum blot. Membranes will be prehybridized with standard hybridization buffer (Boehringer Mannheim) at 55_for 18 hours, hybridized with DIG-labeled antisense RNA at 55_ for 18 hours. Detection was performed with DIG Luminescent Detection Kit (Boehringer Mannheim) according manufacture's instructions. RNAs from S strain isolated from patients with GCA and non GCA will be compared. Comparison will be done manually and by computerized densitometry using 23s rRNA no internal control.

Our results revealed 11 genetic loci expressed significatly higher in strains from patient with gastric cancer. Nested control study suggested a higher risk in patents positive for antibody to the 26kD protein, however, it was not statiscally significant due to limited case number.

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