計畫編號：NHRI-EX95-9520BI

國家衛生研究院整合性醫藥衛生科技研究計畫

B型肝炎病毒pre-S表面抗原突變型引發肝癌的基因不
穩定性

95年度成果報告

執行機構：成功大學

計畫主持人：黃溫雅副教授

本年度執行期間  95 年 1 月 1 日至 95 年 12 月 31 日

＊＊本研究報告僅供參考用，不代表本院意見＊＊
一、95年度計畫研究成果摘要

計畫名稱：B型肝炎病毒pre-S表面抗原突變型引發肝癌的基因不穩定性
計畫編號：NHRI-EX95-9520B1

執行機構：成功大學

計畫主持人：黃溫雅

研究人員：謝逸敏、沈芳晴、黃玉君、謝惠娟

關鍵字：hepatitis B virus, pre-S mutant, ER stress, retino blastoma, JAB1, hepatocellular carcinoma

成果分類：

☑ 癌症基礎與臨床研究（可選填，最多三項）
☑ 分子與基因醫學研究
☐ 臨床研究
☐ 生物技術與藥物研究
☐ 生物統計與生物資訊研究
☐ 醫療保健政策研究
☐ 環境衛生與職業醫學研究
☐ 醫學工程研究
☐ 老年醫學研究
☐ 精神醫學與藥物濫用研究
☐ 疫苗研究
☐ 幹細胞研究
☐ 奈米醫學研究
☐ 其他重要疾病或醫療衛生問題研究

1
(1) 中文摘要

B型肝炎病毒是台灣一個重要的疾病，也是國人肝癌發生的主要原因。B型肝炎表面抗原的表現型式隨著病毒複製情形不同而有所差異。在急性感染期，由主要抗原，也就是小抗原(major/small HBsAg)來表現。這些表面抗原構成病毒粒子的外包殼，而使個病毒大量複製產生並分泌至肝細胞外。然而，當轉變成慢性感染期時，B型肝炎病毒的基因基本上會嵌入宿主的染色體中。以表面抗原而言，將會轉變成大抗原(large HBsAg)的表現。大抗原與小抗原之間的差異在於起始點的不同。大抗原蛋白包含 pre-S 序列，原為小抗原的上游啟動子區域。我們在先前的研究中發現，慢性 B 型肝炎的後期有些表面抗原基因會在 pre-S 區域發生突變，而產生了突變型的大抗原蛋白。這些 pre-S 突變種 B 型肝炎病毒表面抗原較易發生在肝硬化及肝癌的組織，似乎意味著這些 pre-S 表面抗原突變種與 B 型肝炎所引發的肝癌之間有著密切的關係。

本研究著眼於 pre-S 表面抗原突變種引發之基因不穩定性機轉的探討。我們發現 pre-S 表面抗原突變種與 JAB1 蛋白結合，引起了 p27kip 的降解以及抑癌基因 retinoblastoma 的去活化。這些結果證實了 B 型肝炎病毒 pre-S 表面抗原突變種可引發基因不稳定性與細胞週期失調而造成肝細胞的癌化。
The hepatitis B virus (HBV) large surface antigen (LHBS) mutant with deletion at the pre-S$_2$ region accumulates in endoplasmic reticulum (ER) and is associated with HBV-induced hepatocellular carcinogenesis. In this study, we found that the pre-S$_2$ LHBS mutant directly interacts with the Jun activation domain-binding protein 1 (JAB1). Association of pre-S$_2$ LHBS with JAB1 dissociated JAB1 from the JAB1/IRE1 complex in ER. The free (active) JAB1 then translocated into cell nuclei and rendered the Cdk inhibitor p27$^{kip1}$ to cytosolic proteasome for degradation. We also found that the pre-S$_2$ LHBS mutant induced hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (Cdk2), a downstream molecule regulated by P27$^{kip1}$. This effect is partially dependent on ER stress signaling pathway. On the other hand, the pre-S$_1$ mutant LHBS did not interact with JAB1 but induced ER stress as the pre-S$_2$ mutant LHBS did. Through ER stress the pre-S$_1$ mutant LHBS mildly induced JAB1 nuclear localization and p27$^{kip1}$ degradation but not RB hyperphosphorylation. The transgenic mice carrying the pre-S$_2$ mutant HBS gene also exhibited Cdk2 activation, P27$^{kip1}$ degradation as well as RB hyperphosphorylation. The mouse hepatocytes exhibited morphologic abnormalities such as chromatin condensation, multi-nucleation, and dysplasia of hepatocytes. In summary, the pre-S$_2$ LHBS mutant causes RB hyperphosphorylation through direct interaction with JAB1. The pre-S$_2$ mutant LHBS is suggested to be a potential oncoprotein for HBV-related hepatocellular carcinoma.
<table>
<thead>
<tr>
<th>序號</th>
<th>計畫產出名稱</th>
<th>產出型式</th>
<th>Impact factor</th>
<th>致謝對象</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>24. Tsai, P.-C., Huang, W., Lee, Y.-C., Chan, S.-H., and Guo, Y.-L. Genetic polymorphisms in CYP1A1 and GSTM1 predispose humans to PCBs/PCDFs-induced skin lesions. Chemosphere 2006;63:1410-1418. (SCI) Accepted</td>
<td>Foreign</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>27. Wang, H.-C., Lai, M.-D., Huang, W. and Su, I.-J ER stress induces cytoplasmic cyclin A and centrosome overduplication. 2006; Submitted</td>
<td>Foreign</td>
<td>NHRI</td>
<td></td>
</tr>
<tr>
<td>Patent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>序號</td>
<td>計畫產出名稱</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>無</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Book</th>
</tr>
</thead>
<tbody>
<tr>
<td>序號</td>
</tr>
<tr>
<td>無</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conference Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>序號</td>
</tr>
<tr>
<td>無</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Technical Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>序號</td>
</tr>
<tr>
<td>無</td>
</tr>
</tbody>
</table>

2-1
參、95年度計畫重要研究成果產出統計表

<table>
<thead>
<tr>
<th>科技論文篇數</th>
<th>技術</th>
<th>國內</th>
<th>國外</th>
<th>類型</th>
<th>經費</th>
<th>項數</th>
</tr>
</thead>
<tbody>
<tr>
<td>期刊論文</td>
<td></td>
<td>0篇</td>
<td>4篇</td>
<td>技術</td>
<td>千元</td>
<td>項</td>
</tr>
<tr>
<td>研討會論文</td>
<td></td>
<td>2篇</td>
<td>2篇</td>
<td>技術</td>
<td>千元</td>
<td>項</td>
</tr>
<tr>
<td>專著</td>
<td></td>
<td>篇</td>
<td>篇</td>
<td>技術</td>
<td>千元</td>
<td>項</td>
</tr>
<tr>
<td>專利</td>
<td></td>
<td>項</td>
<td>項</td>
<td>技術</td>
<td>千元</td>
<td>項</td>
</tr>
</tbody>
</table>

[註]：

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部份，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專著：為對某項學術進行專門性探討之純學術性作品。

技術報告：指從事物某技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術轉移：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者。
肆、95年度計畫重要研究成果

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

計畫之新發現、新發明或對學術界、產業界具衝擊性(impact)之研究成果，請依性質勾選下列項目。

☑ 1. 研發或改良國人重要疾病及癌症的早期診斷方式及治療技術
☐ 2. 發展新的臨床治療方式
☐ 3. 發展新生物製劑、篩檢試劑及新藥品
☑ 4. 瞭解常見疾病及癌症之分子遺傳機轉
☐ 5. 瞭解抗癌藥劑對癌細胞之作用機制
☐ 6. 提供有效的疾病預防策略
☐ 7. 利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展
☐ 8. 醫療保健政策相關研究
☐ 9. 瞭解環境毒理機制及重金屬對人體健康的影響
☐ 10. 研發適合臨床使用的人造器官及生醫材料
☐ 11. 縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用
☐ 12. 改進現有醫療器材的功能或增加檢驗影像的解析能力
☐ 13. 其他重要疾病或醫藥衛生問題研究
hepatitis B virus pre-S2 mutant surface antigen associates with Jun activation domain-binding protein 1 and induces Retinoblastoma hyperphosphorylation

A. Introduction

Chronic viral hepatitis is the major cause for hepatocellular carcinoma (HCC), the most frequent visceral neoplasm worldwide. Approximately 500,000 new cases are diagnosed with viral hepatitis and almost as many die of it yearly (1). The main causative agents for HCC are HBV and HCV, which together are responsible for about 80% of all HCCs in humans (2-7). These viruses cause necroinflammatory liver disease of variable duration and severity. A major portion of the viral hepatitis progresses into liver cirrhosis and dysplasia, and ultimately HCC (8). In this process the viral proteins are believed to be important players, which cross-talk with various host proteins, affecting the host signaling pathways. A number of HBV and HCV gene products have been identified as viral tumor proteins, such as the HCV core protein, which induces generation of reactive oxygen species and apoptosis, and the E1 and E2 proteins, which cause ER stress and ER-related signaling pathways (9). Among the HBV proteins, the X protein (pX) is oncogenic because it activates the Ras/Raf-1 signal transduction pathway and inhibits DNA repair (10-12). HBV surface protein (HBsAg) expression in the chronic phase of HBV infection is also associated with HCC incidence (13). HBsAg causes sustained hepatic inflammation and injury, an important marker identifying chronic HBV carriers (14).

In the chronic phase of HBV infection, the HBV genome often integrates into the host chromosome (15). In this status, the large form of HBsAg (LHBS)
becomes largely expressed (16, 17). Compared with small-form HBsAg, the large form includes an additional pre-S region that is the upstream promoter region for the small-form (16, 17). LHBS is pro-oncogenic: it induced HCC in a transgenic mouse model (18, 19). These findings indicate that LHBS interacts with host factors to regulate the mechanism of hepatocellular carcinogenesis.

Ground glass hepatocytes (GGHs) types I and II are the histological hallmarks of chronic HBV infection (20-22). Based on immunohistological studies, type I GGHs display an inclusion-like pattern of HBsAg, whereas type II GGHs display HBsAg at the margins (20-22). Subsequent studies indicated that the LHBS genes in these GGHs carry specific mutations (23). Type I GGHs are partially deleted in the pre-S₁ promoter region, whereas type II are deleted in approximately nucleotides 4 to 57 of the pre-S₂ region and often contain a point mutation in the start codon of the region, which leads to a dramatic decrease in the synthesis of small and middle-sized surface antigens (Fig. 1A). Type II GGHs often appear in hepatic nodules and proliferate in clusters, strongly suggesting that they are involved in HBV-related hepatocarcinogenesis (24, 25). This mutant form of the HBS gene, designated pre-S₂ HBS mutant, emerges only in the late or non-replicative phase of chronic HBV infection and eventually becomes a dominant HBV gene product in hepatocytes (24, 25).

The pre-S₂ type of LHBS mutant is predominant in HCC patients with HBV infection (25-29). Based on epidemiological studies, HBV carriers who presented with the pre-S₂ LHBS mutant in serum had worse disease outcomes than those who did not (29, 30). Although the correlation between the prevalence of pre-S₂ LHBS mutant and HCC has been clearly demonstrated, the molecular mechanism of hepatocellular carcinogenesis induced by the pre-S₂ LHBS mutant is not yet clear. In a recent study (31), we found that pre-S₂ LHBS mutants accumulated in ER and induced strong ER stress, which induced oxidative stress, DNA damage, and mutagenesis, all of which resulted in
genomic instability in hepatocytes. The pre-S₂ LHBS mutant also induced overexpression of cyclin A, which is associated with the G1-S cyclin-dependent kinases (Cdk), leading to cell cycle progression in the presence of DNA lesions (32). In the present study, we searched for the molecule directly targeted by the pre-S₂ LHBS mutant and the mechanism for pre-S₂ LHBS mutant-induced hepatocellular carcinogenesis.

B. Results

1. Pre-S₂ LHBS mutant associated with JAB1

We used yeast two-hybrid assays to identify the human protein directly targeted by the pre-S₂ LHBS mutant. We found that Jun activation domain-binding protein (JAB) 1 was associated with the pre-S₂ mutant LHBS (40). In the human hepatoma HuH-7 cells transfected with the wild-type, pre-S₁ or pre-S₂ mutant LHBS, we found that the JAB1 protein was associated with the pre-S₂ LHBS mutant, but not with the wild-type or the pre-S₁ LHBS mutant, shown by co-immunoprecipitation experiments (Fig. 1B and C). JAB1 is an ER factor and activated upon ER stress (41). To confirm that the pre-S mutant LHBS indeed induce ER stress in these cells, the ER stress marker XBP1 mRNA, which is alternatively spliced upon ER stress, was measured (37). The cleaved mRNA product, which was processed into the functional XBP1 protein, was found significantly increased in the cells carrying the pre-S₁ or pre-S₂ LHBS mutant than in those carrying the wild-type LHBS, indicating that the pre-S mutant LHBS induce significant ER stress, as we previously reported (Fig. 1D). One study (41) reported that JAB1 is associated with the ER transmembrane kinase/ribonuclease IRE1α ER lumen, but we found that association has dramatically diminished in cells expressing pre-S₂ LHBS mutant (Fig. 1E, F and G). This suggested that pre-S₂ LHBS mutant interacts with JAB1 and disrupts
the association between JAB1 and IRE1. In the cases of wild-type and pre-S1 LHBS mutant, this disruption is dependent on ER stress, as the ER stress inhibitor vomitoxin could nearly completely recover the association between JAB1 and IRE1 (Fig. 1G). However, in the case of pre-S2 LHBS mutant, the association between JAB1 and IRE1 could not be recovered, indicating that the pre-S2 LHBS mutant disrupted the JAB1-IRE1 binding by specifically interacting with JAB1 (Fig. 1G). Macrophage migration inhibitory factor (MIF) protein has also been associated with JAB1 in cytosol (42). Co-immunoprecipitation studies showed that pre-S2 LHBS mutant also disrupted the association between MIF and JAB1 (Fig. 1H). Therefore, the pre-S2 LHBS mutant appears to competitively bind with JAB1 and dissociate JAB1/IRE1 and JAB1/MIF complexes.

2. Pre-S2 LHBS mutant-induced degradation of Cdk inhibitor p27Kip1

Free (active) JAB1 translocates into cell nuclei, and then targets the Cdk inhibitor p27Kip1 and brings it to proteasome 26S for degradation (43, 44). To confirm that the pre-S LHBS mutants triggered nuclear localization of JAB1, the nuclear and cytosolic fractions were analyzed for JAB1 protein levels. We found that, in the presence of pre-S2 LHBS mutant, nuclear JAB1 levels were consistently significantly higher than those induced by the wild-type (Fig. 2A). On the contrary, the pre-S1 LHBS mutant mildly enhanced the JAB1 nuclear localization. In the continuous time course analyses, nuclear localization of JAB1 was clearly seen 40 hours after transfection of the pre-S1 LHBS mutant gene, but not observed 36 or 48 hours after the transfections (Fig. 2A). These results reveal that the cells carrying the pre-S2 mutant LHBS exhibited JAB1 nuclear localization much more significantly than those carrying the pre-S1 mutant LHBS.

We also found that the pre-S1 and pre-S2 LHBS mutants down-regulated
p27^{Kip1}; however, after treatment with the 26S proteasome inhibitor lactacystin, the loss in p27^{Kip1} protein level was abrogated (Fig. 2B). This revealed that pre-S LHBS mutants activated JAB1 and caused p27^{Kip1} to be degraded via proteolysis. These findings strongly suggest that the pre-S$_2$ LHBS mutant induced p27^{Kip1} degradation mainly through its direct binding to JAB1 and induced significant JAB1 nuclear localization; whereas the pre-S$_1$ LHBS mutant induced JAB1 nuclear localization in much lower level and solely mediated by ER stress.

P27^{Kip1} is an inhibitor for the kinase activity of the cyclin-Cdk2 complex (45). To detect whether the Cdk2 was indeed activated by the pre-S mutant LHBS, the activated Cdk2, indicated by its phosphorylation on the threonine residue at amino acid 160, was detected in HuH-7 cells carrying the wild-type, the pre-S$_1$, or the pre-S$_2$ LHBS mutant (46). It was found that while total Cdk2 levels had not changed in various transfected cells, the phosphorylation on threonine 160 of Cdk2 was significantly greater in cells carrying the pre-S$_2$ LHBS mutant than in cells carrying the wild-type LHBS, while the pre-S$_1$ LHBS mutant induced moderate level of CDK$_{T160}$ phosphorylation (Fig. 2C). On the contrary, the levels of cyclin D$_1$, associated with Cdk4 in the G1-Cdk complex, were not changed in various transfected cells (Fig. 2C) (47). These findings indicate that the pre-S$_2$ LHBS mutant causes activation of the cyclin-Cdk2 complex through p27^{Kip1} degradation.

3. Pre-S$_2$ LHBS mutant induced RB hyperphosphorylation

Cyclin A-Cdk2 complex plays key role for entry of cell cycle S phase by phosphorylating the tumor suppressor protein RB (48). We found that human hepatoma HuH-7 cells with the pre-S$_2$ LHBS mutant, but not those carrying the wild-type or the pre-S$_1$ LHBS mutant, showed RB hyperphosphorylation (Fig. 3A). Such RB hyperphosphorylation was indeed caused primarily by cyclin-dependent kinase (Cdk) 2, which phosphorylates RB at the threonine
residue at amino acid 821 (Fig. 3B) (49). To show that such an effect induced by pre-\(S_2\) LHBS mutant was not cell-type-specific, the T24 human bladder cancer cell line was also used for the present study, and it showed RB hyperphosphorylation by Cdk2 when transfected with the pre-\(S_2\) LHBS mutant gene (Fig. 3C and D), indicating that RB hyperphosphorylation induced by the pre-\(S_2\) LHBS mutant is solely dependent on expression of the pre-\(S_2\) LHBS mutant gene. In addition, the analysis of co-immunoprecipitation experiments showed that the association between RB and \(E_2F_1\) was greatly diminished (Fig. 3E), indicating that pre-\(S_2\) LHBS mutant-induced RB hyperphosphorylation released \(E_2F_1\) from the RB-\(E_2F_1\) complex and potentially caused a G1-to-S transition (50).

4. RB hyperphosphorylation in transgenic mice with the pre-\(S_2\) LHBS mutant

We established an FVB/N mouse model containing the pre-\(S_2\) HBS mutant transgene (31). ELISA analysis detected high levels of HBV surface antigens in the sera of these pre-\(S_2\) HBS transgenic mice (Fig. 4A). Liver tissue showed a large number of HBsAg\(^+\) hepatocytes (Fig. 4B) with abnormal morphologies, i.e., centrosome condensation, aneuploidy, and dysplasia (Fig. 4C). After examining the phosphorylation status of RB in the non-tumorous sections of liver in these mice, we found that the majority of transgenic mice with the pre-\(S_2\) LHBS mutant gene showed RB hyperphosphorylation (Fig. 4D), which was consistent with our findings in human HuH-7 \textit{in vitro} cell cultures. Furthermore, in these liver sections, Cdk2 was activated (shown as the increased phosphorylation of threonine 160 on Cdk2) (Fig. 4D). Similar to the findings in human HuH-7 cells, we found that \(p27^{Kip1}\) protein levels in mouse hepatocytes with the pre-\(S_2\) LHBS mutant transgene were significantly lower than in those with the wild-type LHBS transgene (Fig. 4D). This means that the pre-\(S_2\) LHBS mutant induces RB hyperphosphorylation through \(p27^{Kip1}\) degradation in \textit{in vivo} mouse models.
C. Discussions and impacts

After investigating the carcinogenesis process induced by the pre-S$_2$ LHBS mutant, we found that the pre-S$_2$ LHBS mutant directly interacts with the endoplasmic reticulum factor JAB1 and subsequently results in hyperphosphorylation of the retinoblastoma tumor-suppressor protein. Taking the previous and current findings together, a model was proposed (Fig. 5): in the late phase of chronic HBV infection, the pre-S$_2$ LHBS mutant, emerging through immune escape, accumulates in endoplasmic reticulum and induces strong ER stress and oxidative DNA damage (31). In the ER lumen, the pre-S$_2$ LHBS mutant also interacts with JAB1, dissociating it from the JAB1-IRE1 complex. A fraction of the pre-S$_2$ LHBS mutant also disrupts the JAB1-MIF complex in cytosol. The free JAB1 then translocates into cell nuclei, targeting the Cdk inhibitor p27$^{kip1}$ and bringing p27 to cytosolic 26S proteasomes for degradation. The loss of p27 activates the cyclin A/Cdk2 complex, leading to RB hyperphosphorylation as well as to G1-to-S transition. The combined events of oxidative DNA damage and cell cycle progression consequently result in genomic instabilities of hepatocytes and, ultimately, hepatocellular carcinogenesis.

We found that the JAB1 protein was associated with the pre-S$_2$ LHBS mutant much more strongly than with the wild-type or pre-S$_1$ LHBS mutant. The mechanism for such a differential binding affinity is unknown. The deletion of 54 nucleotides in the pre-S$_2$ region of the pre-S$_2$ LHBS mutant is predicted to affect an N-glycosylation event near the start of the pre-S$_2$ region, based on bioinformatic analysis (data not shown). Therefore the defect of N-glycosylation might cause misfolding of the pre-S$_2$ LHBS mutant and result in conformational distortion, which dramatically changes its binding properties (51). It would be intriguing to compare the structures of the pre-S$_2$ LHBS mutant with those of the wild type and the pre-S$_1$ mutant using protein crystallographic studies.
JAB1 is a multifunctional protein associated with the signaling pathway, cell-cycle regulation, and development, and acts as a key subunit of the COP9 signalosome (CSN) (40). Overexpression of CSN5/JAB1 promotes cell proliferation, increases AP-1 transcription, and stimulates or inhibits turnover of a number of proteins. CSN5/JAB1 also activates JNK kinase activity and increases the c-Jun phosphorylation level (40, 42). We recently found that c-Jun was upregulated by the pre-S₂ LHBS mutant in mouse ML1 hepatoma cells (data not shown), suggesting that the pre-S₂ LHBS mutant indeed triggers activation of the AP1 transcription factor. CSN5/JAB1 binding has also been shown to stimulate protein degradation, whereas in other cases, such as HIF1-, CSN5/JAB1 binding tends to promote stabilization (52). Given the multiple functionalities of JAB1, there might be other pathways of pre-S₂ LHBS mutant-induced carcinogenesis, in addition to p27^Kip1 degradation. It was also reported that JAB1 participates in UPR through its association and dissociation with ER factor IRE1 (41). Therefore, JAB1 also plays an active role in ER stress signaling pathways, which activates the NF B proto-oncogene (53). It has been documented that JAB1 is highly expressed in hepatocellular carcinoma and other common types of cancers, including breast, lung, and ovarian cancers (54-58). And the ER stress signaling pathways likely contribute to the JAB1-related carcinogenesis process.

Previous studies have shown that the pre-S₁/pre-S₂ LHBS mutants induce strong ER stress (25). In the present study, we found that association of pre-S₂-LHBS-mutant with JAB1 is partially dependent on ER stress, because the ER stress inhibitors partially blocked such association, suggesting that the ER stress-signaling pathway is an essential but not sufficient mechanism for the pre-S₂ LHBS mutant-induced JAB1 activation.

ER stress is a consequence of viral replication in mammalian cells (59). The overexpressed viral proteins accumulate in ER lumen, waiting to mature or, if
misfolded, to degrade (59). It is, therefore, conceivable that viral proteins induce temporary ER stress. Thus, the pre-S₂ LHBS mutant is extremely special in that it directly interacts with the ER factor JAB1 and affects ER function. Through its interaction with JAB1, the pre-S₂ LHBS mutant potentially disrupts the process to eliminate the misfolded or unassembled proteins from the ER to the cytosolic ubiquitin-proteasome system (UPS) for degradation (60, 61). Using electron microscopy, we analyzed GGH type II cells, which harbor the pre-S₂ LHBS mutant and saw large amounts of pre-S₂ LHBS mutant stalled in ER, which resulted in ER enlargement (data not shown). This phenomenon could be described as R constipation? This observation has implied that the ER-associated degradation (ERAD) pathway, the main pathway to clear the misfolded proteins from ER lumen, is likely defective (60, 61). In fact, HIV was also recently found to exploit some elements of ERAD, causing strong ER stress (62). Therefore, to fully understand the molecular mechanism(s) of the pre-S₂ mutant LHBS-associated hepatocellular carcinogenesis, it shall be very important to explore the functional relevance of pre-S₂ mutant LHBS with ER signaling pathways, including ERAD.

We previously demonstrated that the pre-S mutants induce strong ER stress-dependent oxidative DNA lesions that activated the DNA repair mechanism and mutagenesis (31). The DNA damage potentially deactivates certain tumor suppressors or proto-oncogenes, leading to cellular transformation and hepatocellular carcinogenesis. The results of the present study have added another important mechanism for pre-S₂-LHBS-mutant-induced hepatocellular carcinogenesis: RB hyperphosphorylation and defects in G1-S cell cycle arrest. Taking these findings together, we hypothesize that in the presence of DNA damage, the failure of cell cycle arrest evidently increases gene mutation rates and genomic instabilities and further enhances the carcinogenesis process (63). Our recent epidemiological studies found that patients who presented with pre-S
LHBS mutant in serum developed HCC at significantly higher rates (odds ratio 3.2, data not shown) than those without. In addition, approximately 60% of HCC patients who were HBV carriers presented with the pre-S LHBS mutant in serum (data not shown). The results of the present study provide an explanation of the mechanism that yields such a high association of pre-S2 LHBS mutant with HCC. Based on our studies, we propose that the pre-S2 LHBS mutant is a potential oncoprotein and may represent an important predictive marker for the HCC caused by chronic HBV infection.

The hepatocellular carcinogenesis caused by HBV is a complicated and multifactorial process. Oncogene c-myc has been proposed to play a role in it (64). The HBV X protein, encoded DNA polymerase for virus replication, is also highly-associated with HCC incidence, because it interacts with the host nucleotide excision repair (NER) factors and down-regulates DNA repair (10-12). How these factors work in concert to initiate/promote HCC is the next important question to address. The findings in these studies would not only elaborate the development of HCC caused by HBV but also benefit that by HCV, because of the similarities of ways of cross-talks between host factors and viral proteins of HBV and HCV (1, 2, 9).

In summary, we found that the pre-S2 LHBS mutant directly interacts with the ER factor JAB1 and triggers p27Kip1 degradation and RB hyperphosphorylation. This is a novel mechanism for hepatocellular carcinogenesis caused by HBV. Based on the results of the present study, we conclude that the pre-S2 LHBS mutant is an important prognostic marker for chronic HBV infection and should be widely applied in clinical medicine.
D. References


53. Kaneko M, Niinuma Y, Nomura Y. Activation signal of nuclear factor-kappa


FIGURE LEGENDS

Figure 1. Association of the ER factor JAB1 with the pre-S₂ mutant large HBsAg. A, Maps for the wild-type, pre-S₁ mutant, and pre-S₂ mutant large HBsAg. The pre-S₁ type of mutant HBsAg is deleted of nucleotides 3040 to 3111 in the pre-S₁ region; whereas the pre-S₂ type is deleted of nucleotides 4 to 57 and contains a point mutation at the beginning of the pre-S₂ region. B, Association of JAB1 with large HBsAg in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1) or pre-S₂ mutant (Δ2) large HBS genes in HuH-7 cells, detected by co-immunoprecipitation assays using the antibody against hemagglutinin (HA) epitope fused to various types of LHBS. The immuno-precipitants were detected by western blots using the antibodies against HA or the JAB1 protein. The cell lysates were detected for the total levels of JAB1 and LHBS, using the antibody specifically recognizing the pre-S₁ region of the LHBS. gp42, the 42 kDa, glycosylated form of LHBS (39 kDa). C, control, cells transfected with the plasmid vector pIRE5-hrGFP-2a only. C, Reciprocal immuno-precipitation analysis to detect the association of pre-S₂ mutant LHBS with JAB1. D, Induction of ER stress by the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg in HuH-7 cells, detected by the alternative splicing patterns of XBP1 mRNA by multiplex RT-PCR analysis. Form I, total XBP1 (unspliced and spliced); form II, spliced XBP1. TG, cells treated with thapsigargin (1 M) for 3 hr; TM, cells treated with tunicamycin (10 g/mL) for 3 hr; C, control, cells transfected with the plasmid vector pIRE5-hrGFP-2a only. E, Association of ER factor IRE1 with JAB1 in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg, shown by co-immunoprecipitation assays using the anti-JAB1 antibody. The immuno-precipitants were detected for the levels of IRE1. The cell lysates were
also detected for the total levels of JAB1 and IRE1. \( F, \) Dissociation of IRE1 with JAB1, induced by the ER stress inducers tunicamycin \((10 \text{ g/mL})\) and thapsigargin \((1 \text{ M})\). The JAB1 levels were detected in the immuno-precipitants containing the IRE1. \( G, \) Reciprocal co-immunoprecipitation experiments to detect the association of ER factor IRE1 with JAB1 in the cells transfected with the wild-type \( (WT)\), pre-\( S_1 \) mutant \( (\Delta_1)\), and pre-\( S_2 \) mutant \( (\Delta_2)\) large HBsAg. The immuno-precipitants containing IRE1 were detected for the JAB1 levels. The cell lysates were also detected for the total levels of JAB1 and IRE1. \( VT, \) vomitoxin \((150 \text{ ng/mL})\), the ER stress inhibitor, was used to detect the dependence of IRE1-JAB1 on ER stress. \( H, \) Association of MIF with JAB1 in the cells transfected with the wild-type \( (WT)\), pre-\( S_1 \) mutant \( (\Delta_1)\), and pre-\( S_2 \) mutant \( (\Delta_2)\) large HBsAg, shown by co-immunoprecipitation assays using the anti-JAB1 antibody.
**Figure 2.** JAB1 subcellular localization and p27Kip1 degradation affected by the pre-S1 and pre-S2 mutant LHBS. **A,** JAB1 levels in the nuclear and cytosolic fractions in the HuH-7 cells 36, 40, and 48 hours post LHBS transfections, shown by western blots. WT, wild-type; Δ1, pre-S1 mutant; Δ2, pre-S2 mutant LHBS. Cdk4 and α-tubulin were used as markers for proteins in nuclei and cytosol, respectively. **C,** control, the un-transfected cells. **B,** Degradation of p27Kip1 in the HuH-7 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg, shown by western blots. LACTA, lactacystin (10 μM), the 26 proteasome inhibitor. **C,** Activation of the Cdk2 in the HuH-7 cells carrying the pre-S mutant LHBS by western blot: The levels of total Cdk2, Cdk4, Cyclin D1, and the Cdk2 phosphorylated at threonine 160 (pCdk2T160) were detected in the cells carrying wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg.
Figure 3. RB hyperphosphorylation by Cdk2 in the HuH-7 cells carrying the pre-S2 mutant large HBsAg. A, Western blot to detect total RB hyper-phosphorylation in the HuH-7 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. C, control, cells transfected with the plasmid vector pIRES-hrGFP-2a only. LHBS, large HBV surface antigen; gp42, the 42 kDa, glycosylated form of LHBS (39 kDa). The pre-S1 and S2 mutant HBS antigens are smaller in sizes than the wild-type HBS is. IB, immunoblot. B, Western blot to detect RB hyperphosphorylation at threonine 821 in the HuH-7 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. The pRB$^{T821}$ was detected with the monoclonal antibody specifically recognizes the RB phosphorylated at threonine 821. EGF, epidermal growth factor, the positive control, which has been shown to activate Cdk2. C, RB hyperphosphorylation in the T24 human bladder cancer cells carrying the pre-S2 mutant large HBsAg. D, Western blot to detect pRB$^{T821}$ hyper-phosphorylation in the T24 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. E, Association of E2F1 with RB in HuH-7 cells carrying wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg by the immunoprecipitation (IP) assays, using the mouse 11D7 anti-RB antibody. The immuno-precipitant was detected for the E2F1 levels. F, RB phosphorylation status after treatments with the ER stress inhibitors TMB-8 (250 μM) or vomitoxin (150 ng/mL) in HuH-7 cells carrying wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. The phosphorylation status on threonine 821 of RB protein was detected by western blots.
Figure 4. RB hyperphosphorylation in the transgenic mice carrying the pre-S₂ mutant LHBS. A, Serum HBS gene and protein levels, detected by PCR and ELISA assays. M, DNA size marker. C, negative control, the PCR reaction without addition of DNA template. pHBV3.6, the plasmid containing the HBV genome DNA, used as a control for HBS gene (658 bp) in PCR reaction. HBS DNA in sera of the transgenic mice carrying the wild-type (WT, 658 bp) or pre-S₂ mutant (Δ2) HBS genes were detected by PCR. The GAPDH (293 bp) is used as internal control. B, Expressions of the wild-type (WT) and pre-S₂ mutant (Δ2) large HBsAg in hepatocytes of the transgenic mice. The brown-colored cells on the arrows are the HBsAg-expressing cells. C, control, the naïve mice. C, Morphologies of the liver sections in the transgenic mice, shown by hematoxylin-eosin staining method. The arrows point to cells that display morphological abnormalities, including nuclear enlargement and chromosome condensation. D, RB and Cdk2 phosphorylations, as well as p27^{Kip1} levels, in the non-tumor liver tissues of representative mice carrying the wild-type (WT) or pre-S₂ mutant (Δ2) HBS transgenes. C, naïve mice. For each detected mouse, the serum HBsAg level (ng/mL), detected by the ELISA assays, was shown at the bottom of its PCR products.
Figure 5. A model of hepatocellular carcinogenesis caused by the HBV pre-S \(_2\) mutant large HBsAg. In the absence of the ER stress or the pre-S \(_2\) mutant HBsAg (top panel), the JAB1 is associated with IRE1 in ER lumen and MIF in cytosol. When the pre-S \(_2\) mutant HBsAg accumulates in ER, it induces strong ER stress (bottom panel). It also directly associates with IRE1 in ER lumen and MIF in cytosol, freeing JAB1 from the JAB1/IRE1 and JAB1/MIF complexes. The free JAB1 translocates into cell nuclei in which it associates with cyclin-dependent kinase inhibitor p27\(^{Kip1}\), bringing p27 to cytosolic 26S proteasome for degradation. Degradation of p27 then activates G1 to S cyclin/Cdk2, which phosphorylates RB, leading to S phase progression. While the cells undergo cell cycle progression, they also suffer from the oxidative DNA damages caused by ER stress; therefore mutagenesis occurs in genomes and ultimately leads cell toward to paths of carcinogenesis.
Wild type: intact S gene

<table>
<thead>
<tr>
<th>Pre-S₁</th>
<th>Pre-S₂</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>2854</td>
<td>3211</td>
<td>155</td>
</tr>
</tbody>
</table>

Δ1: Pre-S₁ deletion

<table>
<thead>
<tr>
<th>Pre-S₂</th>
<th>S</th>
</tr>
</thead>
</table>
- Deletion of nt. 3040-3111 in pre-S₁ region

Δ2: Pre-S₂ deletion

<table>
<thead>
<tr>
<th>Pre-S₁</th>
<th>Pre-S₂</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
- ATG→ATA
- Deletion of nt. 457 in pre-S₂ region

B

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>HA</td>
<td>gp42</td>
<td>p39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LHBS</td>
<td>gp42</td>
<td>p39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### C

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAB1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAB1</td>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAB-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### D

<table>
<thead>
<tr>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
<th>TG</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Form I</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Form II</td>
</tr>
</tbody>
</table>

4-25
### E

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAB1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAB1</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### F

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>TG</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRE1</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRE1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- JAB1
- IRE1
### Fig. 2A

#### 36 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>α-tubulin</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 40 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>α-tubulin</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td><img src="image12.png" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 48 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>α-tubulin</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td><img src="image18.png" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### B

<table>
<thead>
<tr>
<th>LACTA</th>
<th>V</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
<th>V</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- p27
- α-tubulin

### C

<table>
<thead>
<tr>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CDK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK2$^{T160}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4-29
Fig. 3

A

IB  C  WT  Δ1  Δ2

Total RB

pp-RB
pRB

LHBS

gp42
p39

α-tubulin

B

IB  EGF  C  WT  Δ1  Δ2

RB$^{T821}$

α-tubulin
### Table E

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>RB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>E2F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>E2F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>RB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table F

<table>
<thead>
<tr>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
<th>VT</th>
<th>TMB8</th>
<th>W/O FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB T821</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4

A

<table>
<thead>
<tr>
<th></th>
<th>hbb/16.5 Tg</th>
<th>WT- Tg</th>
<th>LHS/Δ2 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2000
1500
1000
900
800
700
600
500
400
300
200
100

658 bp (Wild type)
604 bp (LHS/Δ2)
293 bp (GAPDH)

B

Naïve  WT  Δ2
二、計畫對民眾具教育宣導之研究成果（此部份將為規劃對一般民眾教育或宣導研究成果之依據，請以簡易易懂之文字簡述研究成果，內容以不超過300字為原則）

慢性肝炎、肝硬化及肝細胞癌為國人重要死亡原因，而肝癌更居台灣地區癌症死亡原因的第一位，其中有很大部分是由 B 型、C 型肝炎病毒所引起，其影響至為巨大。我們在先前的研究中發現，慢性 B 型肝炎的後期有些表面抗原基因會在 pre-S 區域發生突變，而產生了突變型的大抗原蛋白。這些 pre-S 突變種 B 型肝炎病毒表面抗原較易發生在肝硬化及肝癌的組織，似乎意味著這些 pre-S 表面抗原突變種與 B 型肝炎所引發的肝癌之間有著密切的關係。本研究結果發現 pre-S 表面抗原突變種會引發基因不穩定性而導致癌症發生。我們發現 pre-S 表面抗原突變種會造成抑癌基因的去活化而加速癌症發生。也許未來我們可以檢測 Pre-S 表面抗原突變種是否存在，來做為慢性 B 型肝炎帶原者轉化成肝癌之指標。
Hepatitis B virus surface antigen pre-S2 mutant causes genomic instabilities in hepatocytes.

1. Hepatitis B virus surface antigen pre-S2 mutant directly interacts with JAB1 protein, resulting in degradation of the cyclin-dependent kinase inhibitor \( p27^{Kip1} \) and phosphorylation of tumor suppressor protein retinoblastoma.

2. Hepatitis B virus surface antigen pre-S2 mutant may serve as a prognostic marker for HBV-induced hepatocellular carcinoma.
四、成效評估（技術面、經濟面、社會面、整合綜效）

The proposed research works and experiments are going along in good progress. The research findings should be able to improve our knowledge for HBV-induced hepatocellular carcinoma. The pre-S2 mutant HBV surface antigen may serve as a good prognostic marker for HBV-induced hepatoma.

五、下年度工作構想及重點之妥適性

The research next year will continue to study the molecular mechanisms for pre-S2 mutant-induced genomic instabilities, as well as to develop therapeutic drugs to block pre-S2 mutant HBV surface antigen to induce hepatoma in chronic HBV carriers.

六、檢討與展望

The research in this year is performed in good progress. We will keep our pace and continue to study the molecular mechanisms of pre-S2 mutant HBV surface antigen-induced hepatoma. We will also focus our efforts on development of drugs specifically targeting on pre-S2 mutant HBV surface antigen.
伍、95年度計畫所培訓之研究人員

<table>
<thead>
<tr>
<th>種 類</th>
<th>人數</th>
<th>備</th>
<th>註</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 博士後研究人員</td>
<td>訓練中</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2. 碩士級研究人員</td>
<td>訓練中</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3. 學士級研究人員</td>
<td>訓練中</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4. 其他</td>
<td>訓練中</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>已結訓</td>
<td></td>
</tr>
<tr>
<td>兼任人員</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 博士班研究生</td>
<td>訓練中</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2. 碩士班研究生</td>
<td>訓練中</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>已結訓</td>
<td></td>
</tr>
<tr>
<td>醫師</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>訓練中</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>已結訓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

特殊訓練課程（請於備註欄說明所訓練課程名稱）
陸，參與95年度計畫所有人力之職級分析

<table>
<thead>
<tr>
<th>職級</th>
<th>所 含 職 級 類 別</th>
<th>參與人次</th>
</tr>
</thead>
<tbody>
<tr>
<td>第一級</td>
<td>研究員、教授、主治醫師</td>
<td>1人</td>
</tr>
<tr>
<td>第二級</td>
<td>副研究員、副教授、總醫師、助教授</td>
<td>1人</td>
</tr>
<tr>
<td>第三級</td>
<td>助理研究員、講師、住院醫師</td>
<td>0人</td>
</tr>
<tr>
<td>第四級</td>
<td>研究助理、助教、實習醫師</td>
<td>1人</td>
</tr>
<tr>
<td>第五級</td>
<td>技術人員</td>
<td>0人</td>
</tr>
<tr>
<td>第六級</td>
<td>支援人員</td>
<td>0人</td>
</tr>
<tr>
<td>合計</td>
<td></td>
<td>3人</td>
</tr>
</tbody>
</table>

[註]：

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、簡任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事、會計、秘書、事務人員及維修、機電人員等。
### 一、參與95年度計畫所有人力之學歷分析

<table>
<thead>
<tr>
<th>類別</th>
<th>學歷</th>
<th>參與人次</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>博士</td>
<td>2人</td>
</tr>
<tr>
<td>2</td>
<td>碩士</td>
<td>1人</td>
</tr>
<tr>
<td>3</td>
<td>學士</td>
<td>1人</td>
</tr>
<tr>
<td>4</td>
<td>專科</td>
<td>0人</td>
</tr>
<tr>
<td>5</td>
<td>博士班研究生</td>
<td>2人</td>
</tr>
<tr>
<td>6</td>
<td>碩士班研究生</td>
<td>2人</td>
</tr>
<tr>
<td>7</td>
<td>其他</td>
<td>3人</td>
</tr>
<tr>
<td></td>
<td>合計</td>
<td>11人</td>
</tr>
</tbody>
</table>

### 二、參與95年度計畫所有協同合作之研究室

<table>
<thead>
<tr>
<th>機構</th>
<th>研究室名稱</th>
<th>研究室負責人</th>
</tr>
</thead>
<tbody>
<tr>
<td>National Health Research Institute</td>
<td>Laboratory of Clinical Virology</td>
<td>Ih-Jen Su</td>
</tr>
</tbody>
</table>


7/8
玖、九十五年度計畫執行情形

注：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

一、請簡述原計畫書中，九十五年預計達成之研究內容

1. Regulation of retinoblastoma (RB) phosphorylation by the pre-S₂ mutant large HBsAg
   a. RB phosphorylation status induced by the pre-S₂ mutant large HBsAg
   b. RB phosphorylation status in transgenic mouse model carrying the pre-S₂ mutant large HBS gene
   c. Regulation of the cyclin/Cdk complexes in cells carrying the pre-S₂ mutant large HBsAg

2. Regulation of ER function and genomic instabilities by the pre-S₂ mutant large HBsAg
   a. Yeast two-hybrid assays to screen for the proteins that are associated with the pre-S₂ mutant large HBsAg
   b. Functional studies of the ER factors that are associated with the pre-S₂ mutant large HBsAg

二、請詳述九十五年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

a. (達成率 100%) RB phosphorylation status induced by the pre-S₂ mutant large HBsAg
b. (達成率 100%) RB phosphorylation status in transgenic mouse model carrying the pre-S₂ mutant large HBS gene
c. (達成率 100%) Regulation of the cyclin/Cdk complexes in cells carrying
the pre-\textit{S}_2 mutant large HBsAg

d. (達成率 100\%) Yeast two-hybrid assays to screen for the proteins that are associated with the pre-\textit{S}_2 mutant large HBsAg

e. (達成率 80\%) Functional studies of the ER factors that are associated with the pre-\textit{S}_2 mutant large HBsAg
拾壹、本年度之著作抽印本或手稿
HEPATITIS B VIRUS PRE-S₂ MUTANT SURFACE ANTIGEN ASSOCIATES WITH JUN ACTIVATION DOMAIN-BINDING PROTEIN 1 AND INDUCES RETINOBLASTOMA HYPERPHOSPHORYLATION

Yi-Hsuan Hsieh¹⁴, Ih-Jen Su⁵,⁶, Hui-Ching Wang⁶, Jui-He Tsai⁴, Wen-Wei Chang¹,², Ming-Derg Lai¹,³,⁵, Huan-Yao Lei¹,², and Wenyua Huang¹,⁴,⁵

¹Departments of ¹Basic Medical Sciences, ²Microbiology and Immunology, ³Biochemistry, ⁴Medical Laboratory Science and Biotechnology, ⁵Center for Gene Regulation and Signal Transduction Research, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan; ⁶Division of Clinical Research, National Health Research Institute, Taipei, Taiwan.

This work was supported by grants from the Program for Promoting University Academic Excellence Project 91-B-FA09-1-4 (Su, I-J) and the National Health Research Institute Extramural Project NHRI-EX95-9520BI (Huang, W), Taiwan.

To whom proofs should be sent: Wenyua Huang, PhD, at Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

70101, Taiwan. Phone: (886) 6-235-3535 ext. 5766; Fax: (886) 6-236-3956; E-mail: whuang@mail.ncku.edu.tw

Running Title: RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

Keywords: hepatitis B virus; pre-S mutant HBsAg; ground glass hepatocyte; ER stress; cyclin-dependent kinase 2; genomic instability; hepatocellular carcinoma; c-Jun activation domain-binding protein 1
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

List of abbreviations:

HBV, hepatitis B virus; LHBS, large hepatitis B virus surface antigen; ER, endoplasmic reticulum; RB, retinoblastoma; JAB1, Jun activation domain-binding protein-1; IRE1, inositol-requiring-1; HCC, hepatocellular carcinoma; sAg, surface antigen; GGH, ground glass hepatocyte; Cdk, cyclin-dependent kinase; ROS, reactive oxygen species; API, activator protein 1; nt, nucleotide; HA, hemagglutinin; TMB-8, 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate; UPR, unfolded protein response; MIF, macrophage migration inhibitory factor; CSN5, COP9 signalosome; JNK, c-Jun amino-terminal kinase; HIF1, hypoxia-induced factor 1; ERAD, ER-associated protein degradation; UPS, ubiquitin-proteasome system; NER, nucleotide excision repair
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

ABSTRACT

The hepatitis B virus (HBV) large surface antigen (LHBS) mutant with deletion at the pre-S2 region accumulates in endoplasmic reticulum (ER) and is associated with HBV-induced hepatocellular carcinogenesis. In this study, we found that the pre-S2 LHBS mutant directly interacts with the Jun activation domain-binding protein 1 (JAB1). Association of pre-S2 LHBS with JAB1 dissociated JAB1 from the JAB1/IRE1 complex in ER. The free (active) JAB1 then translocated into cell nuclei and rendered the Cdk inhibitor p27^{Kip1} to cytosolic proteasome for degradation. We also found that the pre-S2 LHBS mutant induced hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (Cdk2), a downstream molecule regulated by P27^{Kip1}. This effect is partially dependent on ER stress signaling pathway. On the other hand, the pre-S1 mutant LHBS did not interact with JAB1 but induced ER stress as the pre-S2 mutant LHBS did. Through ER stress the pre-S1 mutant LHBS mildly induced JAB1 nuclear localization and p27^{Kip1} degradation but not RB hyperphosphorylation. The transgenic mice carrying the pre-S2 mutant HBS gene also exhibited Cdk2 activation, P27^{Kip1} degradation as well as RB hyperphosphorylation. The mouse hepatocytes exhibited morphologic abnormalities such as chromatin condensation, multi-nucleation, and dysplasia of hepatocytes. In summary, the pre-S2 LHBS mutant causes RB hyperphosphorylation through direct interaction with JAB1. The pre-S2 mutant LHBS is suggested to be a potential oncoprotein for HBV-related hepatocellular
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS carcinoma.
INTRODUCTION

Chronic viral hepatitis is the major cause for hepatocellular carcinoma (HCC), the most frequent visceral neoplasm worldwide. Approximately 500’000 new cases are diagnosed with viral hepatitis and almost as many die of it yearly (1). The main causative agents for HCC are HBV and HCV, which together are responsible for about 80% of all HCCs in humans (2-7). These viruses cause necroinflammatory liver disease of variable duration and severity. A major portion of the viral hepatitis progresses into liver cirrhosis and dysplasia, and ultimately HCC (8). In this process the viral proteins are believed to be important players, which cross-talk with various host proteins, affecting the host signaling pathways. A number of HBV and HCV gene products have been identified as viral tumor proteins, such as the HCV core protein, which induces generation of reactive oxygen species and apoptosis, and the E1 and E2 proteins, which cause ER stress and ER-related signaling pathways (9). Among the HBV proteins, the X protein (pX) is oncogenic because it activates the Ras/Raf-1 signal transduction pathway and inhibits DNA repair (10-12). HBV surface protein (HBsAg) expression in the chronic phase of HBV infection is also associated with HCC incidence (13). HBsAg causes sustained hepatic inflammation and injury, an important marker identifying chronic HBV carriers (14).

In the chronic phase of HBV infection, the HBV genome often integrates into the host
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

chromosome (15). In this status, the large form of HBsAg (LHBS) becomes largely expressed (16, 17). Compared with small-form HBsAg, the large form includes an additional pre-S region that is the upstream promoter region for the small-form (16, 17). LHBS is pro-oncogenic: it induced HCC in a transgenic mouse model (18, 19). These findings indicate that LHBS interacts with host factors to regulate the mechanism of hepatocellular carcinogenesis.

Ground glass hepatocytes (GGHs) types I and II are the histological hallmarks of chronic HBV infection (20-22). Based on immunohistological studies, type I GGHs display an inclusion-like pattern of HBsAg, whereas type II GGHs display HBsAg at the margins (20-22). Subsequent studies indicated that the LHBS genes in these GGHs carry specific mutations (23). Type I GGHs are partially deleted in the pre-S1 promoter region, whereas type II are deleted in approximately nucleotides 4 to 57 of the pre-S2 region and often contain a point mutation in the start codon of the region, which leads to a dramatic decrease in the synthesis of small and middle-sized surface antigens (Fig. 1A). Type II GGHs often appear in hepatic nodules and proliferate in clusters, strongly suggesting that they are involved in HBV-related hepatocarcinogenesis (24, 25). This mutant form of the HBS gene, designated pre-S2 HBS mutant, emerges only in the late or non-replicative phase of chronic HBV infection and eventually becomes a dominant HBV gene product in hepatocytes (24, 25).

The pre-S2 type of LHBS mutant is predominant in HCC patients with HBV infection
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

(25-29). Based on epidemiological studies, HBV carriers who presented with the pre-S2 LHBS mutant in serum had worse disease outcomes than those who did not (29, 30). Although the correlation between the prevalence of pre-S2 LHBS mutant and HCC has been clearly demonstrated, the molecular mechanism of hepatocellular carcinogenesis induced by the pre-S2 LHBS mutant is not yet clear. In a recent study (31), we found that pre-S2 LHBS mutants accumulated in ER and induced strong ER stress, which induced oxidative stress, DNA damage, and mutagenesis, all of which resulted in genomic instability in hepatocytes. The pre-S2 LHBS mutant also induced overexpression of cyclin A, which is associated with the G1-S cyclin-dependent kinases (Cdk), leading to cell cycle progression in the presence of DNA lesions (32). In the present study, we searched for the molecule directly targeted by the pre-S2 LHBS mutant and the mechanism for pre-S2 LHBS mutant-induced hepatocellular carcinogenesis.
MATERIALS AND METHODS

Cell lines and mice. Human hepatoma HuH-7 and bladder cancer T24 cell lines were used for in vitro cell culture studies. These cells were maintained in regular Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1× non-essential amino acid, and 1× antibiotic/antimycotic mixture (GIBCO BRL, Life Technologies, Inc., Grand Island, NY). The cells were grown at 37°C with 5% CO₂.

To create transgenic mice carrying wild-type or pre-S₂ LHBS mutant genes, p(3A)sAg or p(3A)sAg-Δ2 plasmid, which contains the wild-type LHBS gene or the gene with an internal deletion on the pre-S₂ region (nt 4-57), was microinjected into FVB/N mouse embryos (31). Mice carrying the transgenes were confirmed by detecting the pre-S₂ LHBS mutant gene and HBsAg expression in serum (31). Transgenic mice were fed standard laboratory chow and water ad libitum in the animal facility of National Cheng Kung University Medical Center. The animals were raised and cared for according to the guidelines set up by the National Science Council, Taiwan.

Western blotting. The protocols for Western blotting basically followed those described elsewhere (31). The protein products of the large HBsAg were detected using monoclonal
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

antibody raised against the sequence in the pre-S1 region (IgMedica Biotechnology, Taipei, Taiwan). Retinoblastoma (RB) protein was detected using mouse monoclonal antibodies 11D7 and G3-245 (generously supplied by Dr. W-H Lee at the University of California, Irvine), which recognize total RB protein (33, 34). To detect phosphorylated RB T821 and Cdk2 T160, antibodies that specifically recognized the phosphorylated residues were used (AbCam plc, Cambridge, UK, and Novus Biologicals, Inc., Littleton, CO). The other primary antibodies used in this study were mouse monoclonal antibodies against human JAB1 (Becton, Dickenson Biosciences, Palo Alto, CA), MIF (Novus), E3F1, cyclin D1, Cdk2, cyclin A, Cdk4, IRE1, p27kip1, and hemagglutinin (HA) epitope (Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

Co-immunoprecipitation. Protocols for co-immunoprecipitation experiments basically follow those described elsewhere (31). To pull down HBV large HBsAg, mouse anti-HA epitope antibody and protein A/G agarose beads (Santa Cruz) were mixed with cell-free extracts of HuH-7 cells transiently transfected with wild-type or pre-S LHBS mutant genes cloned in pIREShrGFP-2a plasmid (Stratagene, La Jolla, CA), which is tagged with HA epitope. Immunoprecipitants were repeatedly washed with RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, and 5 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), and then analyzed using Western
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

blotting.

*Isolation of nuclear and cytosolic fractions.* Human HuH-7 cells were washed with ice-cold PBS, collected with a cell scraper, and harvested using centrifugation. The cell pellets were lysed in Solution A [50 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitors]. After cell lysis, 10% Triton X was gradually added to the final concentration of 0.5% to extract the cell nuclei. After brief centrifugation, the supernatant (cytosol) was collected. The precipitated cell nuclei were lysed in Solution C [50 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitors] with vigorous vortexing for 15 minutes at 4°C, and then centrifugation. The supernatant, which contained the nuclear extracts, was collected.

*ER stress.* Human HuH-7 cells were treated with an ER stress inducer—tunicamycin (10 μg/mL) or thapsigargin (1 μM)—for 3 hours and then returned to cell medium for 3 hours for further incubation (35, 36). The cells were washed with PBS (pH 7.4), then lysed in RIPA buffer supplemented with protease inhibitors. ER stress was detected by examining the alternative splicing patterns of the ER stress marker XBP1 using multiplex RT-PCR. The amounts of
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

functionally active XBPI, which was unconventionally spliced by IRE1 upon ER-stress, was measured by using the PCR primers for total (un-spliced and spliced) XBPI transcripts, and those specifically for spliced XBPI transcripts (37). The PCR primers used for XBP-1 were:

forward (total), 5'-agcactcagactacgtgcac-3'; reverse (spliced form), 5'-acagagaaaggaggtggt-3';

and reverse (total), 5'-accacattagctgcttc-3' (37).

Western blot analysis for retinoblastoma was conducted using anti-pRB^{381} antibody. To detect the whether pre-S₂ LHBSAg mutant-induced RB hyperphosphorylation is dependent on ER stress, the cells transiently transfected with wild-type or with pre-S mutant genes were treated with ER stress inhibitors—8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (250 μM) or vomitoxin (150 ng/mL) (Sigma-Aldrich)—or mock treated for 24 hours (38, 39). After the cells had been washed with ice-cold PBS (pH 7.4), they were lysed in RIPA buffer and then analyzed using Western blotting.

Pre-S₂ HBS transgenic mice study. Control and pre-S₂ HBS transgenic mice from 12 to 15 months old were killed, and the genomic DNA of each was extracted and tested for the HBS gene using PCR. Expression of HBsAg in mouse serum and hepatocytes was analyzed using ELISA and immunohistochemical tests, respectively. To detect the status of RB phosphorylation, mouse livers were homogenized using a mortar and pestle in ice-cold RIPA buffer supplemented
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

with 2 mM of phenylmethylsulphonylfluoride and protease inhibitor cocktail (Sigma-Aldrich).
The homogenates were transferred to microcentrifuge tubes and then centrifuged at 13,000 rpm for 15 minutes at 4°C. After they had been washed repeatedly, the cell lysates were tested for the phosphorylation status of RB$^{T821}$ and Cdk2$^{T160}$ using Western blotting.
RESULTS

Pre-S₂ LHBS mutant associated with JAB1

We used yeast two-hybrid assays to identify the human protein directly targeted by the pre-S₂ LHBS mutant. We found that Jun activation domain-binding protein (JAB) 1 was associated with the pre-S₂ mutant LHBS (40). In the human hepatoma HuH-7 cells transfected with the wild-type, pre-S₁ or pre-S₂ mutant LHBS, we found that the JAB1 protein was associated with the pre-S₂ LHBS mutant, but not with the wild-type or the pre-S₁ LHBS mutant, shown by co-immunoprecipitation experiments (Fig. 1B and C). JAB1 is an ER factor and activated upon ER stress (41). To confirm that the pre-S mutant LHBS indeed induce ER stress in these cells, the ER stress marker XBP1 mRNA, which is alternatively spliced upon ER stress, was measured (37). The cleaved mRNA product, which was processed into the functional XBP1 protein, was found significantly increased in the cells carrying the pre-S₁ or pre-S₂ LHBS mutant than in those carrying the wild-type LHBS, indicating that the pre-S mutant LHBS induce significant ER stress, as we previously reported (Fig. 1D). One study (41) reported that JAB1 is associated with the ER transmembrane kinase/ribonuclease IRE1 in ER lumen, but we found that association has dramatically diminished in cells expressing pre-S₂ LHBS mutant (Fig. 1E, F and G). This suggested that pre-S₂ LHBS mutant interacts with JAB1 and disrupts the association between
JAB1 and IRE1. In the cases of wild-type and pre-S1 LHBS mutant, this disruption is dependent on ER stress, as the ER stress inhibitor vomitoxin could nearly completely recover the association between JAB1 and IRE1 (Fig. 1G). However, in the case of pre-S2 LHBS mutant, the association between JAB1 and IRE1 could not be recovered, indicating that the pre-S2 LHBS mutant disrupted the JAB1-IRE1 binding by specifically interacting with JAB1 (Fig. 1G). Macrophage migration inhibitory factor (MIF) protein has also been associated with JAB1 in cytosol (42). Co-immunoprecipitation studies showed that pre-S2 LHBS mutant also disrupted the association between MIF and JAB1 (Fig. 1H). Therefore, the pre-S2 LHBS mutant appears to competitively bind with JAB1 and dissociate JAB1/IRE1 and JAB1/MIF complexes.

Pre-S2 LHBS mutant-induced degradation of Cdk inhibitor p27Kip1

Free (active) JAB1 translocates into cell nuclei, and then targets the Cdk inhibitor p27Kip1 and brings it to proteasome 26S for degradation (43, 44). To confirm that the pre-S LHBS mutants triggered nuclear localization of JAB1, the nuclear and cytosolic fractions were analyzed for JAB1 protein levels. We found that, in the presence of pre-S2 LHBS mutant, nuclear JAB1 levels were consistently significantly higher than those induced by the wild-type (Fig. 2A). On the contrary, the pre-S1 LHBS mutant mildly enhanced the JAB1 nuclear localization. In the continuous time course analyses, nuclear localization of JAB1 was clearly seen 40 hours after
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

transfection of the pre-S\textsubscript{1} LHBS mutant gene, but not observed 36 or 48 hours after the transfections (Fig. 2A). These results reveal that the cells carrying the pre-S\textsubscript{2} mutant LHBS exhibited JAB1 nuclear localization much more significantly than those carrying the pre-S\textsubscript{1} mutant LHBS.

We also found that the pre-S\textsubscript{1} and pre-S\textsubscript{2} LHBS mutants down-regulated p27\textsuperscript{Kip1}; however, after treatment with the 26S proteasome inhibitor lactacystin, the loss in p27\textsuperscript{Kip1} protein level was abrogated (Fig. 2B). This revealed that pre-S LHBS mutants activated JAB1 and caused p27\textsuperscript{Kip1} to be degraded via proteolysis. These findings strongly suggest that the pre-S\textsubscript{2} LHBS mutant induced p27\textsuperscript{Kip1} degradation mainly through its direct binding to JAB1 and induced significant JAB1 nuclear localization; whereas the pre-S\textsubscript{1} LHBS mutant induced JAB1 nuclear localization in much lower level and solely mediated by ER stress.

P27\textsuperscript{Kip1} is an inhibitor for the kinase activity of the cyclin-Cdk2 complex (45). To detect whether the Cdk2 was indeed activated by the pre-S mutant LHBS, the activated Cdk2, indicated by its phosphorylation on the threonine residue at amino acid 160, was detected in HuH-7 cells carrying the wild-type, the pre-S\textsubscript{1}, or the pre-S\textsubscript{2} LHBS mutant (46). It was found that while total Cdk2 levels had not changed in various transfected cells, the phosphorylation on threonine 160 of Cdk2 was significantly greater in cells carrying the pre-S\textsubscript{2} LHBS mutant than in cells carrying the wild-type LHBS, while the pre-S\textsubscript{1} LHBS mutant induced moderate level of CDR\textsuperscript{Th160}
phosphorylation (Fig. 2C). On the contrary, the levels of cyclin D1, associated with Cdk4 in the G1-Cdk complex, were not changed in various transfected cells (Fig. 2C) (47). These findings indicate that the pre-S2 LHBS mutant causes activation of the cyclin-Cdk2 complex through p27Kip1 degradation.

Pre-S2 LHBS mutant induced RB hyperphosphorylation

Cyclin A-Cdk2 complex plays key role for entry of cell cycle S phase by phosphorylating the tumor suppressor protein RB (48). We found that human hepatoma HuH-7 cells with the pre-S2 LHBS mutant, but not those carrying the wild-type or the pre-S1 LHBS mutant, showed RB hyperphosphorylation (Fig. 3A). Such RB hyperphosphorylation was indeed caused primarily by cyclin-dependent kinase (Cdk) 2, which phosphorylates RB at the threonine residue at amino acid 821 (Fig. 3B) (49). To show that such an effect induced by pre-S2 LHBS mutant was not cell-type-specific, the T24 human bladder cancer cell line was also used for the present study, and it showed RB hyperphosphorylation by Cdk2 when transfected with the pre-S2 LHBS mutant gene (Fig. 3C and D), indicating that RB hyperphosphorylation induced by the pre-S2 LHBS mutant is solely dependent on expression of the pre-S2 LHBS mutant gene. In addition, the analysis of co-immunoprecipitation experiments showed that the association between RB and E2F1 was greatly diminished (Fig. 3E), indicating that pre-S2 LHBS mutant-induced RB
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

hyperphosphorylation released E2F1 from the RB-E2F1 complex and potentially caused a G1-to-S transition (50).

**RB hyperphosphorylation in transgenic mice with the pre-S2 LHBS mutant**

We established an FVB/N mouse model containing the pre-S2 HBS mutant transgene (31). ELISA analysis detected high levels of HBV surface antigens in the sera of these pre-S2 HBS transgenic mice (Fig. 4A). Liver tissue showed a large number of HBsAg+ hepatocytes (Fig. 4B) with abnormal morphologies, i.e., centrosome condensation, aneuploidy, and dysplasia (Fig. 4C). After examining the phosphorylation status of RB in the non-tumorous sections of liver in these mice, we found that the majority of transgenic mice with the pre-S2 LHBS mutant gene showed RB hyperphosphorylation (Fig. 4D), which was consistent with our findings in human HuH-7 in vitro cell cultures. Furthermore, in these liver sections, Cdk2 was activated (shown as the increased phosphorylation of threonine 160 on Cdk2) (Fig. 4D). Similar to the findings in human HuH-7 cells, we found that p27Kip1 protein levels in mouse hepatocytes with the pre-S2 LHBS mutant transgene were significantly lower than in those with the wild-type LHBS transgene (Fig. 4D). This means that the pre-S2 LHBS mutant induces RB hyperphosphorylation through p27Kip1 degradation in in vivo mouse models.
DISCUSSION

After investigating the carcinogenesis process induced by the pre-S₂ LHBS mutant, we found that the pre-S₂ LHBS mutant directly interacts with the endoplasmic reticulum factor JAB1 and subsequently results in hyperphosphorylation of the retinoblastoma tumor-suppressor protein. Taking the previous and current findings together, a model was proposed (Fig. 5): in the late phase of chronic HBV infection, the pre-S₂ LHBS mutant, emerging through immune escape, accumulates in endoplasmic reticulum and induces strong ER stress and oxidative DNA damage (31). In the ER lumen, the pre-S₂ LHBS mutant also interacts with JAB1, dissociating it from the JAB1-IRE1 complex. A fraction of the pre-S₂ LHBS mutant also disrupts the JAB1-MIF complex in cytosol. The free JAB1 then translocates into cell nuclei, targeting the Cdk inhibitor p27^Kip1 and bringing p27 to cytosolic 26S proteasomes for degradation. The loss of p27 activates the cyclin A/Cdk2 complex, leading to RB hyperphosphorylation as well as to G1-to-S transition. The combined events of oxidative DNA damage and cell cycle progression consequently result in genomic instabilities of hepatocytes and, ultimately, hepatocellular carcinogenesis.

We found that the JAB1 protein was associated with the pre-S₂ LHBS mutant much more strongly than with the wild-type or pre-S₁ LHBS mutant. The mechanism for such a differential binding affinity is unknown. The deletion of 54 nucleotides in the pre-S₂ region of the pre-S₂
LHBS mutant is predicted to affect an N-glycosylation event near the start of the pre-S₂ region, based on bioinformatic analysis (data not shown). Therefore the defect of N-glycosylation might cause misfolding of the pre-S₂ LHBS mutant and result in conformational distortion, which dramatically changes its binding properties (51). It would be intriguing to compare the structures of the pre-S₂ LHBS mutant with those of the wild type and the pre-S₁ mutant using protein crystallographic studies.

JAB1 is a multifunctional protein associated with the signaling pathway, cell-cycle regulation, and development, and acts as a key subunit of the COP9 signalosome (CSN) (40). Overexpression of CSN5/JAB1 promotes cell proliferation, increases AP-1 transcription, and stimulates or inhibits turnover of a number of proteins. CSN5/JAB1 also activates JNK kinase activity and increases the c-Jun phosphorylation level (40, 42). We recently found that c-Jun was upregulated by the pre-S₂ LHBS mutant in mouse MLI hepatoma cells (data not shown), suggesting that the pre-S₂ LHBS mutant indeed triggers activation of the AP1 transcription factor. CSN5/JAB1 binding has also been shown to stimulate protein degradation, whereas in other cases, such as HIF1-α, CSN5/JAB1 binding tends to promote stabilization (52). Given the multiple functionalities of JAB1, there might be other pathways of pre-S₂ LHBS mutant -induced carcinogenesis, in addition to p27^{kip1} degradation. It was also reported that JAB1 participates in UPR through its association and dissociation with ER factor IRE1 (41). Therefore, JAB1 also
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

plays an active role in ER stress signaling pathways, which activates the NFκB proto-oncogene (53). It has been documented that JAB1 is highly expressed in hepatocellular carcinoma and other common types of cancers, including breast, lung, and ovarian cancers (54-58). And the ER stress signaling pathways likely contribute to the JAB1-related carcinogenesis process.

Previous studies have shown that the pre-S1/pre-S2 LHBS mutants induce strong ER stress (25). In the present study, we found that association of pre-S2-LHBS-mutant with JAB1 is partially dependent on ER stress, because the ER stress inhibitors partially blocked such association, suggesting that the ER stress-signaling pathway is an essential but not sufficient mechanism for the pre-S2 LHBS mutant-induced JAB1 activation.

ER stress is a consequence of viral replication in mammalian cells (59). The overexpressed viral proteins accumulate in ER lumen, waiting to mature or, if misfolded, to degrade (59). It is, therefore, conceivable that viral proteins induce temporary ER stress. Thus, the pre-S2 LHBS mutant is extremely special in that it directly interacts with the ER factor JAB1 and affects ER function. Through its interaction with JAB1, the pre-S2 LHBS mutant potentially disrupts the process to eliminate the misfolded or unassembled proteins from the ER to the cytosolic ubiquitin-proteasome system (UPS) for degradation (60, 61). Using electron microscopy, we analyzed GGH type II cells, which harbor the pre-S2 LHBS mutant and saw large amounts of pre-S2 LHBS mutant stalled in ER, which resulted in ER enlargement (data not shown). This
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

phenomenon could be described as "ER constipation". This observation has implied that the ER-associated degradation (ERAD) pathway, the main pathway to clear the mis-folded proteins from ER lumen, is likely defective (60, 61). In fact, HIV was also recently found to exploit some elements of ERAD, causing strong ER stress (62). Therefore, to fully understand the molecular mechanism(s) of the pre-S\textsubscript{2} mutant LHBS-associated hepatocellular carcinogenesis, it shall be very important to explore the functional relevance of pre-S\textsubscript{2} mutant LHBS with ER signaling pathways, including ERAD.

We previously demonstrated that the pre-S mutants induce strong ER stress-dependent oxidative DNA lesions that activated the DNA repair mechanism and mutagenesis (31). The DNA damage potentially deactivates certain tumor suppressors or proto-oncogenes, leading to cellular transformation and hepatocellular carcinogenesis. The results of the present study have added another important mechanism for pre-S\textsubscript{2}-LHBS-mutant-induced hepatocellular carcinogenesis: RB hyperphosphorylation and defects in G1-S cell cycle arrest. Taking these findings together, we hypothesize that in the presence of DNA damage, the failure of cell cycle arrest evidently increases gene mutation rates and genomic instabilities and further enhances the carcinogenesis process (63). Our recent epidemiological studies found that patients who presented with pre-S LHBS mutant in serum developed HCC at significantly higher rates (odds ratio 3.2, data not shown) than those without. In addition, approximately 60% of HCC patients
who were HBV carriers presented with the pre-S LHBS mutant in serum (data not shown). The results of the present study provide an explanation of the mechanism that yields such a high association of pre-S₂ LHBS mutant with HCC. Based on our studies, we propose that the pre-S₂ LHBS mutant is a potential oncoprotein and may represent an important predictive marker for the HCC caused by chronic HBV infection.

The hepatocellular carcinogenesis caused by HBV is a complicated and multifactorial process. Oncogene c-myc has been proposed to play a role in it (64). The HBV X protein, encoded DNA polymerase for virus replication, is also highly-associated with HCC incidence, because it interacts with the host nucleotide excision repair (NER) factors and down-regulates DNA repair (10-12). How these factors work in concert to initiate/promote HCC is the next important question to address. The findings in these studies would not only elaborate the development of HCC caused by HBV but also benefit that by HCV, because of the similarities of ways of cross-talks between host factors and viral proteins of HBV and HCV (1, 2, 9).

In summary, we found that the pre-S₂ LHBS mutant directly interacts with the ER factor JAB1 and triggers p27kip1 degradation and RB hyperphosphorylation. This is a novel mechanism for hepatocellular carcinogenesis caused by HBV. Based on the results of the present study, we conclude that the pre-S₂ LHBS mutant is an important prognostic marker for chronic HBV infection and should be widely applied in clinical medicine.
REFERENCES


pre-S mutants in serum and liver at different replicative stages of chronic HBS infection.

Hapatology 2001;33:277-86.


B virus infection induce oxidative stress and DNA damage. Carcinogenesis 

proliferation of hepatocytes induced by a pre-S2 deletion mutant in chronic HBV infection. 
Hepatology 2005;41:761-70.

33. DeCaprio JA, Ludlow JW, Figge J, et al. SV40 large tumor antigen forms a specific 

retinoblastoma-associated proteins: identification of a gene with properties of the 


36. McDowell W, Schwarz RT. Dissecting glycoprotein biosynthesis by the use of specific 


54. Patil MA, Gutgemann I, Zhang J, et al. Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and JAB1 as a potential target for 8q gain in


RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS


FIGURE LEGENDS

Figure 1. Association of the ER factor JAB1 with the pre-S₂ mutant large HBsAg. A, Maps for the wild-type, pre-S₁ mutant, and pre-S₂ mutant large HBsAg. The pre-S₁ type of mutant HBsAg is deleted of nucleotides 3040 to 3111 in the pre-S1 region; whereas the pre-S₂ type is deleted of nucleotides 4 to 57 and contains a point mutation at the beginning of the pre-S2 region. B, Association of JAB1 with large HBsAg in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1) or pre-S₂ mutant (Δ2) large HBS genes in HuH-7 cells, detected by co-immunoprecipitation assays using the antibody against hemagglutinin (HA) epitope fused to various types of LHBS. The immuno-precipitants were detected by western blots using the antibodies against HA or the JAB1 protein. The cell lysates were detected for the total levels of JAB1 and LHBS, using the antibody specifically recognizing the pre-S₁ region of the LHBS. gp42, the 42 kDa, glycosylated form of LHBS (39 kDa). C, control, cells transfected with the plasmid vector pIRES-hrGFP-2a only. C, Reciprocal immuno-precipitation analysis to detect the association of pre-S₂ mutant LHBS with JAB1. D, Induction of ER stress by the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg in HuH-7 cells, detected by the alternative splicing patterns of XBP1 mRNA by mutiplex RT-PCR analysis. Form I, total XBP1 (unspliced and spliced); form II, spliced XBP1. TG, cells treated with thapsigargin (1 μM) for 3
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

hr; TM, cells treated with tunicamycin (10 μg/mL) for 3 hr; C, control, cells transfected with the plasmid vector pIRES-hrGFP-2a only. E, Association of ER factor IRE1 with JAB1 in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg, shown by co-immunoprecipitation assays using the anti-JAB1 antibody. The immuno-precipitants were detected for the levels of IRE1. The cell lysates were also detected for the total levels of JAB1 and IRE1. F, Dissociation of IRE1 with JAB1, induced by the ER stress inducers tunicamycin (10 μg/mL) and thapsigargin (1 μM). The JAB1 levels were detected in the immuno-precipitants containing the IRE1. G, Reciprocal co-immunoprecipitation experiments to detect the association of ER factor IRE1 with JAB1 in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg. The immuno-precipitants containing IRE1 were detected for the JAB1 levels. The cell lysates were also detected for the total levels of JAB1 and IRE1. V, vomitoxin (150 ng/mL), the ER stress inhibitor, was used to detect the dependence of IRE1-JAB1 on ER stress. H, Association of MIF with JAB1 in the cells transfected with the wild-type (WT), pre-S₁ mutant (Δ1), and pre-S₂ mutant (Δ2) large HBsAg, shown by co-immunoprecipitation assays using the anti-JAB1 antibody.
Figure 2. JAB1 subcellular localization and p27<sup>Kip1</sup> degradation affected by the pre-S<sub>1</sub> and pre-S<sub>2</sub> mutant LHBS. A, JAB1 levels in the nuclear and cytosolic fractions in the HuH-7 cells 36, 40, and 48 hours post LHBS transfections, shown by western blots. WT, wild-type; Δ1, pre-S<sub>1</sub> mutant; Δ2, pre-S<sub>2</sub> mutant LHBS. Cdk4 and α-tubulin were used as markers for proteins in nuclei and cytosol, respectively. C, control, the un-transfected cells. B, Degradation of p27<sup>Kip1</sup> in the HuH-7 cells carrying the wild-type (WT), pre-S<sub>1</sub> mutant (Δ1), and pre-S<sub>2</sub> mutant (Δ2) large HBsAg, shown by western blots. LACTA, lactacystin (10 μM), the 26 proteasome inhibitor. C, Activation of the Cdk2 in the HuH-7 cells carrying the pre-S mutant LHBS by western blot: The levels of total Cdk2, Cdk4, Cyclin D1, and the Cdk2 phosphorylated at threonine 160 (pCdk2<sup>T160</sup>) were detected in the cells carrying wild-type (WT), pre-S<sub>1</sub> mutant (Δ1), and pre-S<sub>2</sub> mutant (Δ2) large HBsAg.
Figure 3. RB hyperphosphorylation by Cdk2 in the HuH-7 cells carrying the pre-S2 mutant large HBsAg. A, Western blot to detect total RB hyper-phosphorylation in the HuH-7 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. C, control, cells transfected with the plasmid vector pIRES-hrGFP-2a only. LHBS, large HBV surface antigen; gp42, the 42 kDa, glycosylated form of LHBS (39 kDa). The pre-S1 and S2 mutant HBS antigens are smaller in sizes than the wild-type HBS is. IB, immunoblot. B, Western blot to detect RB hyperphosphorylation at threonine 821 in the HuH-7 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. The pRB<sup>821</sup> was detected with the monoclonal antibody specifically recognizes the RB phosphorylated at threonine 821. EGF, epidermal growth factor, the positive control, which has been shown to activate Cdk2. C, RB hyperphosphorylation in the T24 human bladder cancer cells carrying the pre-S2 mutant large HBsAg. D, Western blot to detect pRB<sup>821</sup> hyper-phosphorylation in the T24 cells carrying the wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg. E, Association of E2F1 with RB in HuH-7 cells carrying wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large HBsAg by the immunoprecipitation (IP) assays, using the mouse 11D7 anti-RB antibody. The immuno-precipitant was detected for the E2F1 levels. F, RB phosphorylation status after treatments with the ER stress inhibitors TMB-8 (250 μM) or vomitoxin (150 ng/mL) in HuH-7 cells carrying wild-type (WT), pre-S1 mutant (Δ1), and pre-S2 mutant (Δ2) large
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

HBsAg. The phosphorylation status on threonine 821 of RB protein was detected by western blots.
**Figure 4.** RB hyperphosphorylation in the transgenic mice carrying the pre-S₂ mutant LHBS. A, Scrum HBS gene and protein levels, detected by PCR and ELISA assays. M, DNA size marker. C, negative control, the PCR reaction without addition of DNA template. pHBV3.6, the plasmid containing the HBV genome DNA, used as a control for HBS gene (658 bp) in PCR reaction. HBS DNA in sera of the transgenic mice carrying the wild-type (WT, 658 bp) or pre-S₂ mutant (Δ2) HBS genes were detected by PCR. The GAPDH (293 bp) is used as internal control. B, Expressions of the wild-type (WT) and pre-S₂ mutant (Δ2) large HBsAg in hepatocytes of the transgenic mice. The brown-colored cells on the arrows are the HBsAg-expressing cells. C, control, the naïve mice. C, Morphologies of the liver sections in the transgenic mice, shown by hematoxylin-eosin staining method. The arrows point to cells that display morphological abnormalities, including nuclear enlargement and chromosome condensation. D, RB and Cdk2 phosphorylations, as well as p27Kip1 levels, in the non-tumor liver tissues of representative mice carrying the wild-type (WT) or pre-S₂ mutant (Δ2) HBS transgenes. C, naïve mice. For each detected mouse, the serum HBsAg level (ng/mL), detected by the ELISA assays, was shown at the bottom of its PCR products.
Figure 5. A model of hepatocellular carcinogenesis caused by the HBV pre-S₂ mutant large HBsAg. In the absence of the ER stress or the pre-S₂ mutant HBsAg (top panel), the JAB1 is associated with IRE1 in ER lumen and MIF in cytosol. When the pre-S₂ mutant HBsAg accumulates in ER, it induces strong ER stress (bottom panel). It also directly associates with IRE1 in ER lumen and MIF in cytosol, freeing JAB1 from the JAB1/IRE1 and JAB1/MIF complexes. The free JAB1 translocates into cell nuclei in which it associates with cyclin-dependent kinase inhibitor p27^Kip1, bringing p27 to cytosolic 26S proteasome for degradation. Degradation of p27 then activates G1 to S cyclin/Cdk2, which phosphorylates RB, leading to S phase progression. While the cells undergo cell cycle progression, they also suffer from the oxidative DNA damages caused by ER stress; therefore mutagenesis occurs in genomes and ultimately leads cell toward to paths of carcinogenesis.
Fig. 1A

Wild type: intact S gene

<table>
<thead>
<tr>
<th>Pre-S₁</th>
<th>Pre-S₂</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>2854</td>
<td>3211</td>
<td>155</td>
</tr>
</tbody>
</table>

Δ₁: Pre-S₁ deletion

- Deletion of nt. 3040-3111 in pre-S₁ region

Δ₂: Pre-S₂ deletion

<table>
<thead>
<tr>
<th>Pre-S₁</th>
<th>Pre-S₂</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATG→ATA</td>
</tr>
</tbody>
</table>
- Deletion of nt. 4-57 in pre-S₂ region

B

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ₁</th>
<th>Δ₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>HA</td>
<td>gp42 p39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>JAB1</td>
<td>gp42 p39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LHBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
E

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAB1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAB1</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
-     IRE1|  |     |    |    |    |
-     JAB1|  |     |    |    |    |

F

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>TG</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRE1</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRE1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
-     JAB1|  |     |    |    |
-     IRE1|  |     |    |    |
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

G

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRE1</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRE1</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>IRE1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAB-1</td>
<td>JAB-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAB-1</td>
<td>MIF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>MIF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2A

36 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

40 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

48 hrs

<table>
<thead>
<tr>
<th>IB</th>
<th>Whole</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>WT</td>
<td>Δ1</td>
</tr>
<tr>
<td>JAB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

B

<table>
<thead>
<tr>
<th></th>
<th>V</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>V</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CDK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK2\textsuperscript{T160}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

Fig. 3

A

<table>
<thead>
<tr>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RB</td>
<td>pp-RB</td>
<td>pRB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHBS</td>
<td>gp42</td>
<td>p39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>IB</th>
<th>EGF</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB^{T821}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

C

IB C WT Δ1 Δ2
Total RB
pp-RB pRB
LHBS gp42 p39
α-tubulin

D

IB EGF C WT Δ1 Δ2
RB^{T821}
α-tubulin
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

E

<table>
<thead>
<tr>
<th>IP</th>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>RB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>E2F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>E2F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>RB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th>IB</th>
<th>C</th>
<th>WT</th>
<th>Δ1</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB T821</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VT</th>
<th>TMB8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>W/O</td>
</tr>
<tr>
<td>WT</td>
<td>FBS</td>
</tr>
<tr>
<td>Δ1</td>
<td>Δ2</td>
</tr>
</tbody>
</table>
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

Fig. 4

A

B

Naïve  WT  Δ2
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

C

Naive  WT  Δ2

D

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>WT</th>
<th>WT</th>
<th>Δ2</th>
<th>Δ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total RB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK-2T160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HBsAg (ng/mL):
- 4854  4877  488  274
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS

Fig. 5
RB hyperphosphorylation induced by HBV pre-S2 mutant LHBS
Differential Endoplasmic reticulum stress signaling pathways mediated by inducible nitric oxide synthase

Yi-Hsuan Hsieh, Ih-Jen Su, Huan-Yao Lei, Ming-Derg Lai, Wen-Wei Chang, and Wenya Huang

*Corresponding author, Department of Medical Laboratory Science and Biotechnology. Tel: 886-6-235-3535 ext. 5766; Fax: 886-6-236-3956; E-mail: whuang@mail.ncku.edu.tw

Running title: ER stress mediated by iNOS

This work was supported by grants from the Program for Promoting University Academic Excellence Project 91-B-FA09-1-4 (Su, I-J & Huang, W.) and the National Health Research Institute Extramural Project NHRI-EX95-9520BI (Huang, W.), Taiwan.
Differential endoplasmic reticulum stress signaling pathways mediated by inducible nitric oxide synthase

Abstract

Accumulated misfolded proteins in endoplasmic reticulum (ER) activate ER stress-signaling pathways. We previously found that ER stress induces production of reactive oxygen species (ROS) and oxidative DNA damage. In this study, we identified the ER factors that generate ROS molecules. After mouse NIH3T3 cells were treated with either tunicamycin, which blocks protein glycosylation, or thapsigargin, which induces Ca\(^{2+}\) efflux from ER lumen, oxidative stress was induced, as shown by increased ROS levels and overexpression of the oxidative DNA repair gene hOGG1. We found inducible nitric oxide synthase (iNOS) was involved in the generation of oxidative stress induced by ER stress. When thapsigargin-treated cells were pre-treated with iNOS inhibitors 1400W or L-canavanine, their ER stress-induced oxidative stress was almost totally abolished. However, this effect was not seen in the cells treated with tunicamycin. Therefore, iNOS appears to mediate the ER stress subpathway caused by Ca\(^{2+}\) efflux. To the contrary, after we treated the cells with the 26S proteasome inhibitors lactacystin or MG-132, the UPR-induced oxidative stress dramatically increased, indicating that clearing misfolded proteins from the ER lumen reduced the oxidative stress. Therefore, the oxidative stress
induced by ER stress signaling is mediated through both iNOS-dependent and -independent subpathways.

**Keywords:** ER stress; hOGG1; inducible nitric oxide synthase; oxidative stress; reactive oxygen species; thapsigargin; tunicamycin
Introduction

Endoplasmic reticulum is the major organelle for protein folding and post-translational modification (PTM). The protein misfolding caused by gene mutations or PTM defects results in the accumulation of proteins in ER and induces ER stress, which is processed through a number of signaling pathways, including the unfolded protein response (UPR), the ER overload response (EOR), and steroid uptake [1,2]. The UPR activates inositol requiring enzyme (IRE) 1, ER kinases, and X-box-binding protein (XBP) 1 by alternative splicing [3-5]. The EOR causes Ca\textsuperscript{2+} efflux from the ER lumen and reactive oxygen ion (ROI) release from mitochondria, resulting in oxidative stress and NF\textsubscript{k}B nuclear localization and activation [1]. Through these pathways, ER stress induces cell cycle arrest and apoptosis [2,6]. The proteins accumulated in the ER lumen are eventually exported by retro-translocation to cytosolic 26S proteasome to be degraded [7-9].

We previously found that the ER stress induced by tunicamycin or thapsigargin caused oxidative stress, indicated by an increase in reactive oxygen species (ROS) levels [10]. Tunicamycin blocks protein glycosylation, activating UPR [11]. Thapsigargin, however, increases ER membrane permeability and causes Ca\textsuperscript{2+} efflux, which mimics EOR [12]. Treatments with either tunicamycin or thapsigargin induced oxidative DNA damage. These findings suggested two possible mechanisms: the UPR and EOR are processed through a
common downstream pathway to generate ROS, or the UPR and EOR induce oxidative stress through distinct pathways.

The Ca\(^{2+}\) efflux caused by thapsigargin activated inducible nitric oxide synthase (iNOS) which is potentially the primary inducer of ROS production [13]. Whether the UPR induces iNOS expression was not clear, however. In the present study we investigated the mechanisms of ROS production through the signaling pathways affected by the UPR and EOR. We clarified the role of iNOS on UPR- and EOR-induced oxidative stress.
Materials and methods

Cell line

We used NIH3T3 (mouse embryonic fibroblast) cell line for this study. The cells were maintained at 37°C with 5% CO₂ in regular Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acid, and 1% antibiotic/antimycotic mixture (Gibco BRL, Grand Island, NY).

ROS measurement

ROS levels in cells were measured by fluorescent staining using DCFH-DA, which senses ROS and emits fluorescence [14]. Cells were harvested and re-suspended in PBS (pH 7.4) at a density of 2 x 10^5 cells/ml. For each measurement, 500 μl of cell suspension was incubated with 5 μM of 2', 7'-dichlorodihydrofluorescein (DCFH-DA; Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C. After the reactions, the cells were washed twice with PBS (pH 7.2) and then analyzed using flow cytometry at the wavelength of 530 nm.

Comet Assays

Levels of oxidative DNA damage were measured using single-cell alkaline electrophoretic comet assays [10]. After treatment with tunicamycin (10 μg/mL) or
thapsigargin (1 μM), the NIH3T3 cells were harvested using trypsinization, centrifuged, and resuspended in PBS; cell numbers were then counted using a hemacytometer. To prepare a glass slide for comet assays, PBS (pH 7.4) with 1% agarose was first coated on the slides, and then covered with 1.5% low-melting-point agarose gel in which $4 \times 10^4$ cells were embedded. Next, another layer of 1% low-melting-point agarose gel was placed on top. After the coatings, the slides were covered with cover slips and placed on ice for 5 min. The cells on slides were then lysed with ice-cold lysis buffer (5 M NaCl, 100 mM EDTA, 100 mM Tris-HCl, 1% triton X-100, 10% dimethylsulfoxide) at 4°C for 1 h. After lysis, the slides were immersed in PBS (pH 7.4) for 10 min and then subjected to enzymatic digestion.

To detect 8-hydroxyguanine DNA lesions, FPG DNA repair enzyme was used to specifically cleave the DNA strand at 8-hydroxyguanine sites [15]. For these slides, 2 units of FPG were incubated with the slides at 37°C for 1 h. After FPG treatment, the slides were washed a few times in PBS (pH 7.4). For single-cell alkaline electrophoresis, the slides were first immersed in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 min and then subjected to electrophoresis at 23 V and 30 mA for 25 min. After alkaline electrophoresis, the slides were transferred to a neutralization solution of 0.4 M Tris-HCl (pH 7.5) and then stained with SYBR green II for 3 min in the dark. Next, 100 cells on each slide were examined using fluorescence microscopy and scored for the fluorescent tail
length from the cell nuclei. Each cell was assigned a score on an arbitrary five-point scale (0 = no DNA damage; 4 = extensive DNA damage), based on comet tail length migration. The total comet score for each slide equaled the sum of the grades for each evaluated cell (maximum possible score: 400) [10].

Expression of iNOS and OGG1 gene

The mouse NIH3T3 cells were treated with tunicamycin (10 μg/mL) or thapsigargin (1 μM) for 3 h or else mock-treated then returned to DMEM culture medium for 0, 2 or 4 h before harvest. The expression levels of iNOS or OGG1 genes were detected by RT-PCR on mRNAs and western blots on proteins. The reaction of reverse transcription was performed in a mixture of 1 μg of cellular RNA, 0.5 μg of random hexamer, 2 mM of each dNTP, 0.6 μl of RNase inhibitor (Takara Corp., Tokyo, Japan), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Promega). The reaction was performed at 37°C for 90 min and then stopped at 95°C for 5 min.

For each PCR reaction, 1 μl of cDNA product of the reverse transcription reaction was used as the template. The PCR primers used were as follows: iNOS, forward: 5'-gtgttccaccaggagatgttg-3', and reverse: 5'-ctcctgcccactgattctgctc-3'; OGG1, forward: 5'-cactgcactgtgcatccgagg-3', and reverse: 5'-gctgtgcaagccaggctgag-3' [10]. The PCR mix
contained 25 mM N-[Tris (hydroxymethyl) methyl]-3-aminopropanesulfonic acid (TAPS, pH 9.3), 50 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 200 μM of each dNTP, 0.25 μg/μl activated calf thymus DNA, and 2 units of SuperTherm Fold DNA polymerase (JMR Holdings, Kent, UK). The PCR reaction was performed in the following order: 94°C for 10 min, then 35 cycles with each cycle of 94°C for 60 s, 54°C for 45 s, and 72°C for 120 s. Finally, the PCR tubes were incubated at 72°C for 10 min. The PCR products were examined using agarose gel electrophoresis.

*Western blots*

Protocols for western blots basically followed those described in our previous report [16]. The NIH3T3 cell lysates were run by SDS-PAGE electrophoresis then transferred to PVDF membrane by using a Mini Tran-Blot Electrophoretic Transfer Cell (Bio-Rad). The proteins on the membrane were probed with the mouse anti-GRP78 antibody (Santa Cruz) in 1:5000 dilution, the mouse anti-iNOS antibody (Parmingen Transduction Laboratories) in 1:1000 dilution, or the rabbit anti-hOGG1 antibody (BD Bioscience) in 1:1000 dilution, followed with incubations with anti-mouse/rabbit secondary antibodies. Finally, an ECL Western Blotting Detection kit (Amersham) was employed to detect the signals of GRP78, hOGG1 or iNOS proteins.
Southwestern immunoblot

The mouse NIH3T3 cells were treated with tunicamycin (10 μg/mL), thapsigargin (1 μM) or hydrogen peroxide (800 μM) for 3 h or else mock-treated, then subjected to genomic DNA extraction. The genomic DNA was then extracted using standard phenol/chloroform methods and diluted to 1 g/L in 0.4 N NaOH to denature the DNA strands [16]. Five micrograms of genomic DNA from each set of cells was spotted on the Hybond-N+ membrane (Amersham) using the slot blot filtration manifold unit (Amersham). The remaining DNA lesions caused by ER stress were then probed using a goat anti-8-hydroxyguanine antibody (Chemicon), based on the western blot protocol described in our previous report [16]. Finally, signals of the 8-hydroguanine were detected using an ECL Western Blotting Detection kit (Amersham).

iNOS activity

NIH3T3 cells were pre-treated with iNOS inhibitors I400W (10 μM), L-canavanine (1 mM) or aminoguanidine (100 μM) or else mock-treated for 2 h, followed with treatments with the ER stress inducers thapsigargin (1 μM) or tunicamycin (10 μg/mL) for 3 h [17-19]. After the treatments, cells were recovered for 6 h at 37°C in DMEM growth media. To measure iNOS activities, which created nitric oxide and released it extracellularly, the supernatant DMEM media were collected and detected by using Total Nitric Oxide Assay
Kit (R&D Systems, Minneapolis, MN), following the users’ instructions.

**NFκB activation**

To measure NFκB activations, the NIH3T3 cells were first transfected with pNFκB-Luc reporter plasmid (Clontech), which contains NFκB promoter upstream of the luciferase reporter gene [20]. After transfections, the cells were treated with 1400W (10 μM) for 2 h, followed with treatments with tunicamycin (10 μg/mL) or thapsigargin (1 μM) for 3 h. Luciferase activities in the cells were then measured using the Dual-Luciferase Reporter Assay System (Promega).
Results

*ER stress caused oxidative stress*

We found that both tunicamycin and thapsigargin caused oxidative stress in mouse NIH3T3 cells. The ROS levels, measured using DCFH-DA fluorescent staining, were significantly higher after treatments with tunicamycin or thapsigargin (Fig. 1B). Comet assays and Southwestern immunoblots showed that such treatments also caused oxidative DNA damage as the levels of 8-hydroxyguanine, the commonest DNA lesion caused by oxidative stress, increased (Fig. 1C and D). The overexpression of the human 8-oxoguanine glycosylase 1 (hOGG1) gene showed that DNA repair activation for oxidative DNA damage was upregulated (Fig. 1E), suggesting that the tunicamycin- or thapsigargin-induced ER stress causes oxidative DNA damage [8].

*Thapsigargin-induced oxidative stress regulated by iNOS*

We found that tunicamycin and thapsigargin significantly upregulated iNOS gene expression, shown by increase of the iNOS mRNA and protein levels (Fig. 2A and B). And iNOS inhibitors 1400W and L-canavanine greatly decreased the ROS levels enhanced by thapsigargin but not tunicamycin (Fig. 2C and E). This indicates that thapsigargin-induced ROS production is mediated through iNOS; whereas that induced by tunicamycin is not. The iNOS inhibitors also almost completely abolished thapsigargin-induced hOGG1
overexpression, which indicated that iNOS mediated thapsigargin-induced oxidative DNA damage (Fig. 2D and F). However, the iNOS inhibitors did not appear to abolish tunicamycin-induced hOGG1 upregulation, which suggested that iNOS mediated tunicamycin-induced oxidative stress as a result of protein misfolding (Fig. 2D and F). To quantitatively detect the iNOS activities affected by thapsigargin and tunicamycin, total nitric oxide (NO) levels were measured. It was found that thapsigargin greatly enhances cellular NO levels, which were nearly completely diminished by pre-treatments with the iNOS inhibitors 1400W or L-canavanine (Fig. 2G). In the case of tunicamycin, iNOS activity almost returned to basal levels at measurement, which was taken place after 6 hours of recovery. These findings indicate that iNOS mediated the ER stress-induced oxidative stress caused by thapsigargin but not tunicamycin. The NFκB activation, a known marker for iNOS induction, was also found in cells treated by thapsigargin but not tunicamycin (Fig. 2H). Such NFκB activation was nearly completely abolished by 1400W treatment, indicating that iNOS mediates thapsigargin-induced oxidative stress.

To further test whether thapsigargin-induced ER stress is dependent on iNOS, we detected the levels of GRP78, an ER chaperone, as markers for ER stress. We found that the iNOS inhibitor 1400W released cells from thapsigargin-induced ER stress within 3 hours after 1400W treatment. Tunicamycin-induced ER stress, however, remained unchanged (Fig. 2I). Taken together, these findings indicate that thapsigargin, but not tunicamycin, induces
ER stress, which triggers oxidative stress, is dependent on iNOS activity.

Tunicamycin-induced oxidative stress regulated by protein degradation

We hypothesized that the accumulation of misfolded proteins in ER is the main cause of tunicamycin-induced oxidative stress. To test this hypothesis, cells pre-treated with tunicamycin or thapsigargin were further treated with the 26S proteasome inhibitors lactacystin or MG-132. Lactacystin or MG-132 treatments greatly enhanced the ROS levels in tunicamycin- but not thapsigargin-pre-treated cells (Fig. 3A and C). Lactacystin or MG-132 treatments also caused significant increases in expressions of hOGG1 in tunicamycin- but not thapsigargin-pre-treated cells (Fig. 3B and D). It was also found that the 26S proteasome inhibitors lactacystin and MG-132 did not affect iNOS activities enhanced by thapsigargin or tunicamycin (Fig. 3E), indicating that the oxidative stress enhanced by inhibition of protein degradation is not mediated by iNOS. These findings correlated with ROS levels, indicating that efficient degradation of misfolded proteins is essential to relieve cells from oxidative stress caused by tunicamycin but not thapsigargin. And the degradation of misfolded proteins reduced oxidative stress caused by UPR.
Discussion

We found that ER stress is processed through iNOS-dependent and independent pathways. The ER overload response, which induces Ca^{2+} efflux and ROI production, is mediated through iNOS, but the unfolded protein response, which activates ER kinases and chaperones, is not [1,2]. Taking our findings and those of other researchers together it suggests the model in Fig. 6: Accumulated misfolded proteins in the ER lumen induce oxidative stress through two major pathways. The first pathway is the ER overload response, which activates iNOS and induces ROI production. It also subsequently causes NFκB nuclear translocation and activates various downstream signaling molecules such as inflammatory cytokines, which induces more NO production [20,21]. The second pathway is the unfolded protein response, which generates ROS largely through oxidative protein synthesis [4]. The accumulated junk proteins are then transported to the cytosolic 26S proteasome to be degraded, which relieves oxidative stress. Thus, in either case, the ER stress-induced ROS attack DNA, which both damages DNA and upregulates DNA repair [10].

The ER molecules that directly activate iNOS in the ER overload response and unfolded protein response have not yet been identified and will be the next important target of research. A recent study reported [22] that reticulon 3 (RTN3/HAP), a molecule on the
ER membrane, upregulated iNOS and protected cells against ER-specific apoptosis. Whether RTN3/HAP is involved in both the ER overload response and unfolded protein response pathways also needs to be clarified.

iNOS is a pleiotropic signaling molecule [23]. It is highly likely that iNOS or NO interacts with other components of the stress response activated by depletion of the intracellular calcium stores, inhibition of protein glycosylation, or glucose starvation [24-26]. Further studies on the potential iNOS involvement in these pathways are needed to verify this hypothesis.

In addition to ER factors, mitochondria are involved in ER stress-induced ROS accumulation [27-29]. Mitochondria contribute significantly to lethal levels of ROS during periods of sustained ER stress through typical ROS production rates associated with respiration [30]. Alternatively, ER stress or increased ROS may, in turn, signal or cause mitochondrial dysfunction, resulting in increased rates of ROS production [31]. It was recently found that iNOS modulated mitochondrial respiration and mitochondria-dependent apoptosis [32]. Based on these findings, iNOS might mediate the signaling pathways between ER and mitochondria.

The ER stress has been shown to contribute to carcinogenesis process [33-35]. A recent study [36] reported that ER stress induced p53 cytoplasmic localization and blocked p53-dependent apoptosis, suggesting that ER stress inhibited apoptosis. We have also found
that a number of in vitro cultured human cells displayed ER stress-induced p53 cytoplasmic localization (data not shown). In the present study, we found that ER stress induced strong oxidative stress and DNA damages, which evidently cause genomic instability, a pre-requisite for carcinogenesis. These results were consistent with a recent report in that ER stress caused p53 inactivation through DNA damage [37]. Whether the ER stress-induced p53 inactivation is mediated by iNOS or NO is not clear and would be the next interesting question to be addressed. Thus, these findings have offered a potential mechanism for why some over-expressed viral proteins accumulated in ER trigger carcinogenesis in the infected host cells [10,38,39].

ER stress caused by protein accumulation is directly associated with a number of developmental and neurological diseases, including cystic fibrosis and Alzheimer's disease [4]. In this study we found that the accumulation of misfolded proteins in ER caused strong oxidative stress and DNA damages. We have also observed that in the liver and kidneys of the mice treated with tunicamycin, the oxidative stress was apparently strong and cells were severely damaged (data not shown). These findings suggested that protein misfolding and accumulation in the ER lumen caused not only the shortage of functionally normal proteins but also extensive tissue damage, which caused apoptosis and necrosis. To prevent these problems, therapeutic approaches to ER stress-associated diseases should include strategies to release or degrade these non-functional proteins in addition to supplementing functional
analogs of the missing proteins [40,41]. Increased ER-associated protein degradation efficiency in these patients should also be a potential therapeutic approach.

In conclusion, the present study showed that ER-stress-induced oxidative stress is mediated through iNOS-dependent and -independent pathways. These findings can be applied to approaches to diminish ER-stress-induced oxidative stress. They also provide insights for alternate therapeutic strategies for diseases caused by protein misfolding.
Abbreviations: ER, endoplasmic reticulum; EOR, ER overload response; IRE-1, inositol-requiring enzyme-1; FPG, formamidopyrimidine-DNA glycosylase; hOGG1, human 8-oxoguanine glycosylase; ROS, reactive oxygen species; TG, thapsigargin; TM, tunicamycin; TMB-8, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate; UPR, unfolded protein response
ER stress mediated by iNOS

References


ER stress mediated by iNOS

Figure legends

**Fig. 1.** Oxidative DNA damages caused by ER stress inducers tunicamycin or thapsigargin. The NIH3T3 cells were treated with tunicamycin (TM, 10 μg/mL), thapsigargin (TG, 1 μM), hydrogen peroxide (800 μM) or else mock-treated (C, control) for 3 hours before harvest. (A) Induction of ER stress marker GRP78 gene, shown by western blot. (B) Levels of reactive oxygen species, detected by DCFH-DA staining, combined with flow cytometry. H₂O₂, the positive control. (C) Levels of oxidative DNA lesions, detected by comet assays. Open boxes, comet scores (mean ± 1 S.D.) before the FPG treatment; the dotted boxes, the comet scores after the FPG treatment. (D) Amounts of 8-hydroxyguanine, detected by Southwestern immunoblot. (E) Expression levels of the human oxoguanine glycosylase (hOGG) 1 gene, detected by RT-PCR and western blots.

**Fig. 2.** Thapsigargin-induced oxidative stress regulated by iNOS. (A) iNOS mRNA levels, detected by RT-PCR. The NIH3T3 cells were treated with tunicamycin (TM, 10 μg/mL) or thapsigargin (TG, 1 μM) for 3 hours then recovered for 0, 2, or 4 h. C: control, the mock-treated NIH3T3 cells. (B) iNOS protein levels, detected by western blots. The cells were treated with tunicamycin, thapsigargin, or mock-treated (C, control) then recovered for 4 h before harvest. (C) ROS levels, detected by DCFH-DA staining, combined with flow cytometry. The cells were pre-treated with l400W (10 μM) or mock-treated (solvent,
absolute ethanol) for 2 h, followed with treatments with thapsigargin or tunicamycin for 3 h before harvest. (D) hOGG1 mRNA levels in NIH3T3 cells, detected by RT-PCR. The cells were pre-treated with 1400W (bottom panel) or mock-treated (top panel) for 2h, followed with treatments with tunicamycin or thapsigargin for 3 h. The cells were sat in growth medium for 0, 2 or 4 hours before harvest. (E) ROS levels, detected by DCFH-DA staining, combined with flow cytometry. The cells were pre-treated with L-canavanine (1 mM) or mock-treated (solvent, PBS (pH 7.4)), followed with treatments with tunicamycin or thapsigargin for 3 h before harvest. (F) hOGG1 mRNA levels, detected by RT-PCR. The cells were pre-treated with L-canavanine (bottom panel) or mock-treated (top panel) for 2h, followed with treatments with tunicamycin or thapsigargin for 3 h. The cells were sat in growth medium for 0, 2 or 4 hours before harvest. (G) iNOS activities in NIH3T3 cells. The cells were pre-treated with 1400W (10 \(\mu\)M), L-canavanine (1 mM) or mock-treated for 2h, followed with treatments with tunicamycin (TM, 10 \(\mu\)g/mL) or thapsigargin (TG, 1 \(\mu\)M) for 3 h. After the treatments, cells were recovered for 6 h. And the supernatant culture media were collected for NO measurement. (H) NF\(\kappa\)B activation, measured by using the pNF\(\kappa\)B-Luc reporter plasmid. After transfection with pNF\(\kappa\)B, the cells were treated with 1400W or mock-treated for 2h, followed with treatments with tunicamycin or thapsigargin for 3 h. The cells were then harvested and measured for luciferase activities. (I) ER stress affected by iNOS inhibitor 1400W, measured by expression levels of the ER chaperone
ER stress mediated by iNOS

GRP78. The cells were treated with 1400W or mock-treated for 2h, followed with treatments with tunicamycin or thapsigargin for 3 h then analyzed for GRP78 levels by western blots.

Fig. 3. Tunicamycin-induced oxidative stress regulated by 26S proteasome-mediated protein degradation. (A) ROS levels in the NIH3T3 cells after treatments of tunicamycin or thapsigargin in the presence of 26S proteasome inhibitor lactacystin, detected by DCFH-DA fluorescent staining, followed with flow cytometry. The cells were treated with tunicamycin (TM, 10 μg/mL), thapsigargin (TG, 1 μM) or mock-treated for 3 h, followed with treatment with lactacystin (10 μM) or else mock-treated (solvent, absolute ethanol) for 3 h before harvest. (B) hOGG1 mRNA levels, detected by RT-PCR. The cells were treated with tunicamycin, thapsigargin or mock-treated for 3 h, followed with treatment with lactacystin (LACTA, 10 μM) or else mock-treated for 3 h before harvest. (C) ROS levels in the cells after treatments of tunicamycin or thapsigargin in the presence/absence of 26S proteasome inhibitor MG-132. The cells were treated with tunicamycin, thapsigargin or mock-treated for 3 h, followed with treatment with MG-132 (1 μM) or else mock-treated (solvent, DMSO) for 3 h before harvest. (D) hOGG1 mRNA levels, detected by RT-PCR. The cells were treated with tunicamycin, thapsigargin or mock-treated for 3 h, followed with treatment with MG-132 or else mock-treated for 3 h before harvest. (E) iNOS activities in NIH3T3
ER stress mediated by iNOS

cells. The cells were treated with tunicamycin, thapsigargin or mock-treated for 3 h, followed with treatment with MG-132, lactacystin or solvents for 3 h. After the treatments, cells were recovered for 6 h then the supernatant growth media were collected for NO measurement.

Fig. 4. The proposed model for oxidative stress induced by ER stress. Thapsigargin causes efflux of Ca$^{2+}$, which induces iNOS up-regulation and ROI production. It then causes NFκB activation and nuclear translocation, which then further activates iNOS and causes oxidative stress and DNA damages. On the other hand, tunicamycin induces UPR and causes accumulation of misfolded proteins in the ER lumen. These misfolded proteins are then exported out of ER by retro-translocation to cytosolic 26S proteasome to be degraded. Through this process, the oxidative stress resulted from UPR is released.
ER stress mediated by iNOS

Fig1.

A

- TG  TM

GRP78
β-actin

B

![](chart.png)

C

![](chart.png)

□ without fpg  □ with fpg
ER stress mediated by iNOS

Fig. 2

A

<table>
<thead>
<tr>
<th>hr</th>
<th>TG</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

iNOS

β-actin

B

C

iNOS

β-actin

C

Fold ROS

0 2 4 6 8

C TG TM C TG TM

1400W
ER stress mediated by iNOS

D

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th></th>
</tr>
</thead>
</table>
| TM  | -  | -  | -  | -  | +  | +  | +  |*
| hr  | -  | 0  | 2  | 4  | 0  | 2  | 4  |*

oggl

β-actin

I400W

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th></th>
</tr>
</thead>
</table>
| TM  | -  | -  | -  | -  | +  | +  | +  |*
| hr  | -  | 0  | 2  | 4  | 0  | 2  | 4  |*

oggl

β-actin

E

![Graph showing Fold ROS]
Fig. 3

A

![Graph showing ROS fold changes with Lactacystin treatment]

B

<table>
<thead>
<tr>
<th>LACTA</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TG</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

![PCR gel images showing ogg1 and hprt expression]

C

![Graph showing ROS fold changes with MG-132 treatment]
ER stress mediated by iNOS

D

<table>
<thead>
<tr>
<th></th>
<th>TM</th>
<th></th>
<th></th>
<th></th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>0</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>-</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

oggl1

β-actin

MG132

<table>
<thead>
<tr>
<th></th>
<th>TM</th>
<th></th>
<th></th>
<th></th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>0</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>-</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

oggl1

β-actin

E

C

\[ \square C \square TG \square TM \]

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>MG-132</th>
<th>LACTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold iNOS activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

38
Streptococcal pyrogenic Exotoxin B Cleaves the Human
S-adenosylhomocysteine Hydrolase and Induces Hypermethioninemia

Ju-Fei Hsu, Woei-Jer Chuang, Shu-Chu Shiesh, Ching-Chuan Liu, Yee-Shin Lin
Chih-Chieh Wang, Tzu-Fun Fu, Jui-He Tsai, Wei-Lun Tsai, Jiunn-Jong Wu,
Ming-Ter Lin, and Wenyu Huang

1Medical Laboratory Science and Biotechnology; 2Biochemistry; 3Pediatrics;
4Microbiology and Immunology; 5Center for Gene Regulation and Signal Transduction
Research, College of Medicine, National Cheng Kung University, Tainan 70101;
6Medical Sciences, Tzu-Chi University, Hualien, Taiwan.

Running title: cleavage of S-adenosylhomocysteine hydrolase by SPE B
FOOTNOTE

Financial Statement

The authors do not have any financial or other association that might post a conflict of interest.

Financial Support

This work was supported by the National Health Research Institute grant NHRI-EX95-9429SP (Lin, Y-S), Taiwan

Wenya Huang, PhD, to whom correspondence should be addressed. Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, No. 1 University Road, Tainan 70101, Taiwan Tel: 886-6-235-3535 ext. 5766; Fax: 886-6-236-3956; E-mail: whuang@mail.ncku.edu.tw
ABSTRACT

The *Streptococcus pyogenes* (group A *Streptococcus*, GAS) is a common pathogen for pharyngitis, impetigo, and myositis. It also causes severe streptococcal toxic shock syndrome (STSS), which is lethal in humans. Streptococcal pyrogenic exotoxin B (SPE B), the most predominant exotoxin secreted by GAS, is highly associated with disease severities. SPE B is a cysteine protease and matures itself by autocatalysis. Using yeast two-hybrid screening assays, we found that the SPE B was directly associated with the human S-adenosylhomocysteine hydrolase (AdoHcyase), an essential factor for delayed-type immune response. AdoHcyase protein levels and enzymatic activities were significantly higher in human cells infected with the *S. pyogenes* SW510 speB mutant strain than in those infected with the NZ131 wild-type strain. SPE B also inactivated AdoHcyase, shown by the lower levels of homocysteine, the main product of AdoHcyase. We also found SPE B-induced hypermethioninemia, caused by a defect of AdoHcyase, *in vivo* and *in vitro*. Using *in vitro* cleavage assays on recombinant AdoHcyase protein, we found that AdoHcyase is a substrate of SPE B cysteine protease. SPE B, therefore, potentially causes immunosuppression by cleaving AdoHcyase.

Keywords: group A *Streptococcus, Streptococcus pyogenes*, SPE B, cysteine protease,
Cleavage of AdoHcyase by SPE B

S-adenosylhomocysteine hydrolase, yeast two-hybrid, homocysteine
The abbreviations used are: AdoHcyase, S-adenosylhomocysteine hydrolase; DZA, 3-deazaadenosine; GAS, group A Streptococcus; IdeS, IgG-degrading enzyme of Streptococcus pyogenes; SPE, Streptococcal pyrogenic exotoxin; STSS, streptococcal toxic shock syndrome
INTRODUCTION

Group A *Streptococcus* is the most invasive class of streptococcal infection. It is caused primarily by *S. pyogenes* infection, which causes symptoms from mild pharyngitis, to acute rheumatic fever and lethal streptococcal toxic shock syndrome [1]. Unlike other groups of *Streptococci*, GAS exhibits β-hemolysis and a strong capsule made of hyaluronic acid, and a strong antigen pilus M protein, which inhibits the phagocytic activity of the host macrophage, thereby shielding GAS from host immune surveillance [2].

*S. pyogenes* infects hosts through a number of paths: nasal, pharyngeal, and dermal [3]. Different *S. pyogenes* strains likely exhibit different preferential entry sites in hosts, because the bacteria contain the virulence factors responsible for specifically recognizing and attaching to host tissues [3]. The streptococcal pyrogenic exotoxins act as superantigens that stimulate T cells by binding class II MHC molecules directly, which causes a massive detrimental cytokine release [4]. The exotoxin-producing strains have been associated with a toxic shock-like syndrome and other forms of invasive disease associated with severe tissue destruction [5].

SPE B is the most important streptococcal pyrogenic exotoxin in GAS. It is highly associated with disease severity because of its proteolytic activities [6]. Through autocatalytic cleavage, SPE B matures itself into an active cysteine protease, degrades
extracellular matrix proteins fibronectin and vitronectin and increases bacterial attachment
to host cells [7, 8]. It cleaves and activates matrix metalloproteases 2 and 9, which increase
bacterial dissemination [9]. It also causes severe host inflammations by activating
interleukin 1β [10]

In addition to its ability to cleave host proteins, SPE B exerts proteolytic activity on
streptococcal surface proteins, including IgG-binding proteins, thereby avoiding
complement activation [11]. It also inhibits the phagocytic activity of macrophages, but the
mechanism it uses is not clear [12, 13]. To fully understand the mechanisms of SPE B-
conferred immunomodulation and GAS dissemination, functional association of SPE B and
the host factors involved in related pathways need to be identified. In this study, we
screened for the human proteins associated with SPE B and explored their functional
relevance to GAS pathogenesis.
MATERIALS AND METHODS

Bacterial strains, cell lines, and mice - The GAS M49 bacterial strain NZ131 was used for in vitro cell culture as well as in vivo mouse studies. SW510, the isogenic speB null strain was constructed in our previous study [14]. The human kidney fibroblast 293T cells were used for in vitro cell culture studies. The GAS bacteria were grown in media. These cells were maintained in regular Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL). The cells were grown at 37°C with 5% CO₂.

BALB/c mice were given standard laboratory chow and water ad libitum in the animal facility of National Cheng Kung University Medical Center. The mice were raised and cared for according to the guidelines set up by the National Science Council of the Republic of China.

Yeast two-hybrid assays - The speB (C192S) gene was PCR amplified from GAS wild-type A20 strain and cloned into yeast two-hybrid bait plasmid pGBKKT7, which contains the Gal4 DNA-binding domain and was tagged with c-Myc epitope [ ]. The speB (C192S)/pGBKKT7 was then transformed into AH109 yeast strain (MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1 UAS::GAL1 TATA::HIS3, GAL2 UAS::GAL2 TATA::ADE2, URA3::MEL1 UAS::MEL1 TATA::LacZ) by lithium acetate method [ ]
and grown in selective quadruple dropout media [ ]. The expression of SPE (C192S) protein in yeast was detected by western blot using the anti-c-Myc antibody (Santa Cruz Biotechnology). To screen for the SPE B-interacting proteins in humans, the human yeast two-hybrid testis cDNA library in pACT2 (BD Clontech), which contains the Gal4 activation domain, was large-scale transformed into AH109 cells containing the SPE B bait plasmid. The HIS3 gene, which was under the control of Gal4 promoter, was used as the primary screening marker for protein-protein interactions. The HIS3\(^{+}\) yeast colonies were then lifted onto Whatman 3MM filter paper, lysed by repeated freeze-thaw procedures, then immersed in X-gal substrate overnight. The blue colonies, which contained the human protein interacting with SPE B (C192S), were further analyzed.

The positive plasmids were isolated from yeast cells by electroporating yeast DNA into DH5\(\alpha\) E. coli strain then selected with ampicillin. The ampicillin-resistant colonies were subjected to plasmid isolation. The plasmids were grouped by restriction mapping then full-length sequenced. The resulting DNA sequences were subjected to NCBI Blast Search to identify the respective genes.

\textbf{GAS infection} – The overnight bacterial cultures of NZ131 (wild-type) or SW510 (spe B) strains were washed twice with PBS (pH 7.4) then re-suspended to \(\text{O.D}_{600} = 1 \times 10^8\) CFU/mL). For \textit{in vitro} infection models, the human 293T cells were first washed twice with PBS (pH 7.4) then re-suspended in DMEM cell culture medium without FBS. The bacterial
suspensions were mixed with human cells at the multiplicity of infection (MOI) 100 then incubated for 90 min. After bacterial infections, the 293T cells were washed with PBS then re-suspended in DMEM growth media supplemented with penicillin G 10 (µg/mL) and gentamycin (200 µg/mL) and incubated at 37°C for 90 min to kill the bacteria outside the human cells. To evaluate the bacterial infection efficiencies, the 293T cells infected with GAS were also serially diluted and plated on bacterial plates for bacterial colony counting.

*Protein-protein association using a sepharose affinity column* – The human 293T cell-free extracts were passed through a CNBr-activated sepharose 4B column (Pharmacia Biotech) pre-immobilized with the 42-kDa pre-mature form of recombinant SPE B (C192S) protein [15]. The protein-column complexes were thoroughly washed with 10 mM Tris.HCl (pH 7.0) and then eluted by boiling for 5 mins. Using western blotting, we analyzed how much AdoHcyase the eluates contained.

*Homocysteine measurement* – The human 293T cells were infected with NZ131 (WT) or SW510 (spe B) at MOI 100 for 24 hours. The cell media were tested for homocysteine levels using Homocysteine Enzyme Immunoassay (EIA) kit (Axis-Shield AS, Germany). Twenty-five microliters of cell culture medium or mouse plasma was applied to the reaction chamber and processed following the manufacturer instructions. The homocysteine levels were detected by the absorbance at 450 nm, using an ELISA reader (Tecan Sunrise).
concentrations of homocysteine were calculated based on a standard curve made from a number of homocysteine samples in known concentrations.

*Methionine measurement* - Analyzing methionine in samples was done using a method modified from a previous report [16]. Briefly, a single 0.33-mm (1/16-inch) disk, which contained approximately 7.6 μL of blood, was punched from dried blood specimens into a 96-well microtiter plate (Evergreen Scientific) on a No. 505H rack. 200 μL of internal working solution, which contained internal amino acid standards in 50% methanol, was added to each well. The racks were gently shaken on a orbital shaker for 20 min. 100 μL of extract was transferred to a clean microtiter plate (Evergreen Scientific) then dried by using an EvapArray nitrogen evaporator (Jones Chromatography). 80μL of derivatization reagent (3 N HCl in n-butanol) was added to each well, which was then covered and heated to 60 °C for exactly 15 min then dried. 100μL of a 1:1 (by volume) solution of acetonitrile–deionized water containing 0.2 mL/L formic acid was added to each well, which was then sealed with a 96-well Micromat plate cover (Sun Brokers) then analyzed by API 2000™ LC/MS/MS (Applied Biosystems). Concentrations of the methionine were calculated against its deuterated internal standard.

*Recombinant AdoHcyase expression and purification* - The full-length AdoHcyase gene
concentrations of homocysteine were calculated based on a standard curve made from a number of homocysteine samples in known concentrations.

*Methionine measurement* - Analyzing methionine in samples was done using a method modified from a previous report [16]. Briefly, a single 0.33-mm (1/16-inch) disk, which contained approximately 7.6 μL of blood, was punched from dried blood specimens into a 96-well microtiter plate (Evergreen Scientific) on a No. 505H rack. 200 μL of internal working solution, which contained internal amino acid standards in 50% methanol, was added to each well. The racks were gently shaken on a orbital shaker for 20 min. 100 μL of extract was transferred to a clean microtiter plate (Evergreen Scientific) then dried by using an EvapArray nitrogen evaporator (Jones Chromatography). 80μL of derivatization reagent (3 N HCl in n-butanol) was added to each well, which was then covered and heated to 60 °C for exactly 15 min then dried. 100μL of a 1:1 (by volume) solution of acetonitrile–deionized water containing 0.2 mL/L formic acid was added to each well, which was then sealed with a 96-well Micromat plate cover (Sun Brokers) then analyzed by API 2000™ LC/MS/MS (Applied Biosystems). Concentrations of the methionine were calculated against its deuterated internal standard.

*Recombinant AdoHcyase expression and purification* - The full-length AdoHcyase gene
Cleavage of AdoHcyase by SPE B

was PCR amplified from the human 293T cells and cloned into the bacterial pET21b vector. The *E. coli* Rosetta (DE) pLys strain containing the AdoHcyase construct was grown at 37°C in M9 growth medium (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 17 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂ and % glucose) and then treated with 0.5 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 90 mins to induce the expression of AdoHcyase. The cells were transferred to lysis buffer (20 mM Tris.HCl, 200 mM NaCl, pH 8.0) then lysed by liquid shear using a French Press. The bacterial lysates were then centrifuged at 12,000 rpm for 30 min. The protein supernatants were collected and analyzed using 12% SDS-PAGE and then Coomassie Blue staining. The recombinant AdoHcyase protein was purified using Ni²⁺-chelating chromatography (Amersham Pharmacia) with a gradient of 20 to 300 mM imidazole, followed with the BioGel P-30 gel filtration chromatography (136 × 1.2 cm, bed vol 135 ml) using phosphate-buffered saline (pH 7.4) to elute the protein fractions, which were flown through at the rate of 3 mL/h. The protein fractions were stored at -80°C until use.

In vitro *SPE B cleavage assay* - Ten micrograms of the purified recombinant AdoHcyase protein was mixed with 0.1 µg of recombinant SPE B in the ratio of 100:1 in the presence of PBS (pH 7.4) with 5 mM of EDTA and 5 mM of dithiothreitol. The reaction was incubated at 37°C for the designated time intervals ranging from 0 to 24 h. The solution
was then heated at 100°C for 10 min and analyzed using 12% SDS-PAGE, followed with Coomassie blue staining.

**AdoHcyase activity** - Approximately 700 μg of cell lysates from human 293T cells infected with NZ131 (wild-type) or SW510 (speB) strains were mixed with 100 μM adenosylhomocysteine and 4 units of adenosine deaminase (Sigma) in the presence of reaction buffer (10 mM NaH₂PO₄/Na₂HPO₄ and 1 mM EDTA, (pH 7.4)) and incubated at 37°C for 30 min. The reaction products were analyzed using reverse-phase HPLC (Beckman) and a 254-nm UV detector. The AdoHcyase activities in the samples were calculated based on the S-adenosylhomocysteine standards with known concentrations.

**Streptococcus pyogenes infection to mice** - BALB/c mice were infected with NZ131 (wild-type) or SW510 (speB mutant) strains. The mice were anesthetized using ether inhalation and then subcutaneously injected with 2 ml of air to form an air pouch. A bacterial suspension (0.3 ml; 10⁹ CFU/ml) in PBS (pH 7.4) was inoculated into the air pouch. Twenty-four hours after the infection, the mice were killed and their blood was collected from the heart.
RESULTS

Using yeast two-hybrid screening assays, we found that the AdoHcyase interacted with the *S. pyogenes* exotoxin SPE B. To confirm that this interaction occurs *in vivo*, the human 293T cell free extracts were subjected to the sepharose 4B column immobilized with the recombinant SPE B protein. The AdoHcyase in cell free extracts bound to the sepharose beads that had been immobilized with SPE B but not to the beads that had not immobilized with SPE B, indicating that SPE B directly interacts with the human AdoHcyase *in vivo* (figure 1A).

Using western blot analysis, we found that the levels of AdoHcyase protein were reduced in cells infected with the wild-type NZ131 strain, but not in those infected with the isogenic *speB* null strain SW510 (figure 1B). In addition, AdoHcyase enzymatic activity, which hydrolyzes *S*-adenosylhomocysteine to *S*-adenosnine and homocysteine, was also impaired by the wild-type but not the *speB* mutant *S. pyogenes* strains (figure 1C). These findings suggested that SPE B cleaves AdoHcyase and thus impairs its enzymatic activity.

In the cell growth media of the human 293T cells infected with the *S. pyogenes* wild-type NZ131 strain, the levels of homocysteine, the major product of AdoHcyase, was dramatically decreased, compared with those infected with the SW510 *speB* mutant strain (figure 2A). In the mice infected with the wild type NZ131 strain, the homocysteine levels
in plasma were dramatically decreased and significantly lower than in the mice infected with the speB mutant SW510 strain (figure 2B). Although the bacterial infection in the mice induced mild hemolysis, which caused minor false-positive absorbances in the EIA assays, the homocysteine levels in the NZ131-infected and SW510-infected mouse plasma were significantly different. This indicated that SPE B impaired AdoHcyase function and reduced homocysteine levels.

The defect of AdoHcyase caused its substrate S-adenosylhomocysteine and the upstream molecule methionine to accumulate in cells. Using tandem mass spectrometry analysis, we found higher blood methionine levels in the mice infected with wild-type NZ131 strain, than in the mice infected with the speB mutant SW510 strain (figure 2C). These findings indicated that SPE B did indeed inhibit the enzymatic activity of AdoHcyase in vitro and in vivo.

We hypothesized that the SPE B cysteine protease cleaves AdoHcyase and inactivates it. Recombinant AdoHcyase was overexpressed in E. coli and purified to near homogeneity (figure 3A). The AdoHcyase was mixed with recombinant SPE B in the ratio of 100 to 1 for in vitro cleavage assays. We found that the full-length AdoHcyase was cleaved within 30 min after adding SPE B. And within 210 min, the AdoHcyase was non-specifically degraded; whereas the reactions with AdoHcyase or SPE B alone kept the full-length respective proteins (figure 3B). These data indicated that SPE B cleaved AdoHcyase and
Cleavage of AdoHcyase by SPE B

ultimately caused its degradation.
DISCUSSION

In this study, we elaborated the pathogen-host interaction of the *S. pyogenes* exotoxin SPE B. We found that SPE B directly interacted with and cleaved human AdoHcyase, an essential enzyme for nucleic acid biosynthesis [17]. AdoHcyase is also a long known drug target molecule for immunosuppression [18]. Therefore, by cleaving AdoHcyase, SPE B is assumed to suppress host immune surveillance and facilitate bacterial dissemination. Taking together the findings in this and previous studies, we developed a model to graph the effects of SPE B on host AdoHcyase (figure 4): SPE B exotoxin is either released from the GAS that enters the host cells or enters the cells via the receptor [19, 20]. Upon entering the host cells, SPE B interacts with and cleaves AdoHcyase, destroying its enzymatic activity. The loss of AdoHcyase activity downregulates homocysteine levels and causes the substrate *S*-adenosylhomocysteine, and its precursors, *S*-adenosylmethionine and methionine, to accumulate in cells.

AdoHcyase controls intracellular levels of *S*-adenosylhomocysteine and regulates protein and nucleic acid methylation processes [17]. The potent AdoHcyase inhibitors 3-deazaadenosine (DZA) and DZ2002 specifically bind to AdoHcyase, resulting in the accumulation of *S*-adenosylhomocysteine and *S*-adenosylmethionine [18, 21]. Through this process, it inhibits lymphocyte-mediated cytolysis, TNF-α expression, all
Cleavage of AdoHcyase by SPE B

immunosuppressive and anti-inflammatory effects [22]. Based on these findings, we believe that when SPE B cleaves AdoHcyase, it causes DZA-like effects and attenuates host immune functions.

It is not clear whether hypo-homocysteinemia and hyper-methioninemia, caused by defects of AdoHcyase, affect the progression by *S. pyogenes* infection. It is, however, well known that hyper-homocysteinemia is associated with inflammation and immune activation [23]. Therefore, we suggest that the decrease in homocysteine levels at the initial stage of bacterial infection suppresses immune activation and helps bacterial survival. In addition, SPE B and another GAS exotoxin IdeS, cleave and degrade immunoglobulins extracellularly, via their cysteine protease activities to block the host’s innate immune response from phagocytosing the GAS [24]. Through these processes, these exotoxins facilitate GAS invasion and augment the severity of symptoms.

GAS causes a wide spectrum of symptoms in various host organ systems [1, 3]. It is now believed that through differential interactions with different host proteins or receptors in various tissues, GAS triggers a distinct development of symptoms [25, 26]. It was recently found that the patterns of virulence gene expression differed between biofilm and tissue communities of *Streptococcus pyogenes* [27]. Therefore, to identify the pathogen-host relationships in various tissues, genome-wide transcriptomic or proteomic analyses in each affected tissue is necessary. This study identifies an SPE B-host protein interaction using a
Cleavage of AdoHcyase by SPE B

yeast two-hybrid screening assay, a well established proteomic approach. We believe that more such studies will greatly improve our understanding of GAS pathogenesis in different host tissues.
REFERENCES


7. Tsai PJ, Kuo CF, Lin KY. et al. Effect of group A streptococcal cysteine protease on


FIGURE LEGENDS

Figure 1. Association of SPE B with AdoHcyase. (A) Western blot to detect affinity of AdoHcyase to the SPE B coupled to activated sepharose beads. C, input control, the cell free extracts. Un-bound, the human 293T cell fractions not bound to the sepharose beads coated with (SPE B un-bound) or without (un-bound) recombinant SPE B proteins. Bound, the cell fractions bound to the beads coated with (SPE B bound) or without (bound) recombinant SPE B proteins. (B) Expression levels of AdoHcyase in 293T cells infected with *S. pyogenes* NZ131 (wild-type) or SW510 (*speB* mutant) strains. Human AdoHcyase is 70 and 80 kDa in size. Tubulin: loading control. (C) AdoHcyase activities in human 293T cells infected with NZ131 (wild-type) or SW510 (*speB*) strains.

Figure 2. AdoHcyase enzymatic activity affected by SPE B. (A) Homocysteine levels in culture media of the human 293T cells infected with *S. pyogenes* NZ 131 (wild-type) and SW 510 (*speB*) strains. ** indicates that the homocysteine levels in the cells infected with NZ131 and SW510 are significantly different (*p < 0.05*). The data represent the mean values of four independent experiments (+ 1 × S.D.). (B) Homocysteine levels in plasma of the mice infected with *S. pyogenes* NZ131 (wild-type) and SW510 (*speB*) strains. (C) Methionine levels in the blood of the mice infected with *S. pyogenes* NZ131 (wild-type) and
SW510 (speB) strains.

**Figure 3. Cleavage of AdoHcyase by SPE B.** (A) Expression and purification of recombinant AdoHcyase. M, ProSieve Color Protein Markers (Cambrex). Lane 1: *E. coli* Rosetta cell extract only. Lane 2: the *E. coli* transformed with AdoHcyase/pET21b, no IPTG addition. Lane 3: *E. coli* transformed with AdoHcyase/pET21b and then treated with IPTG (0.5 mM) for 90 min. Lane 4: the AdoHcyase/pET21b *E. coli* protein fraction not bound to nickel-chelating column. Lane 5: the AdoHcyase/pET21b *E. coli* protein fraction bound to the nickel-chelating column. Lane 6: AdoHcyase protein fractionated using a P30 gel filtration column. (B) *In vitro* cleavage assay of SPE B-cleaved AdoHcyase. Ten micrograms of purified AdoHcyase was mixed with 0.1 μg of recombinant SPE B then incubated for 30' (30'), 90 (90'), 210 (210') and 450 min (450'), and 24 h. AdoHcyase (24 h), AdoHcyase (5 μg, 47 kDa) only after 24 h in the same reaction condition. SPE B: recombinant SPE B (3 μg) only after 24 h in the same reaction condition. The SPE B was auto-cleaved into the mature 28-kDa product. The arrows are on the protein products of SPE B-cleaved AdoHcyase. M: size marker.

**Figure 4. Model for the SPE B regulation on human AdoHcyase.** SPE B is released from GAS in the host cells or enters the cells via a receptor-mediated process. In the host cells,
Cleavage of AdoHcyase by SPE B

SPE B interacts with and cleaves AdoHcyase, destroying its enzymatic activity. The loss of AdoHcyase activity decreases homocysteine levels and causes the substrate \( S \)-adenosylhomocysteine and its precursors, \( S \)-adenosylmethionine and methionine, to accumulate in cells.
Cleavage of AdoHcyase by SPE B

Fig. 1

A

<table>
<thead>
<tr>
<th>C</th>
<th>un-bound</th>
<th>bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoHcyase</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

SPE B un-bound SPE B bound

- 80 kDa
- 70 kDa

B

<table>
<thead>
<tr>
<th>C</th>
<th>NZ131</th>
<th>SW510</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoHcyase</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- 80 kDa
- 70 kDa

<table>
<thead>
<tr>
<th>Tubulin</th>
<th><img src="image5.png" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 kDa</td>
</tr>
</tbody>
</table>
Cleavage of AdoHcyase by SPE B

![Bar chart showing AdoHcyase activity (µmol/min/mg) for Control, NZ131, and SW510. The chart indicates a significant difference between NZ131 and SW510 with an asterisk (*).](chart.png)
Fig 2

A

B
Cleavage of AdoHcyase by SPE B

![Bar chart showing methionine levels in control, NZ131, and SW510 samples. The chart includes error bars and a significance level marked with **.](image)
Cleavage of AdoHcyase by SPE B

Fig. 3

A

\[
\begin{array}{cccccc}
 & M & 1 & 2 & 3 & 4 & 5 & 6 \\
118 & & & & & & & \\
80 & & & & & & & \\
52 & & & & & & & \\
41 & & & & & & & \\
27 & & & & & & & \\
20 & & & & & & & \\
\end{array}
\]

B

\[
\begin{array}{cccccc}
 & 30' & 90' & 210' & 450' & 24hr & 24hr \\
M & & & & & & & \\
52 & & & & & & & \leftarrow 47\text{kDa} \\
41 & & & & & & & \leftarrow 28\text{kDa} \\
27 & & & & & & & \\
20 & & & & & & & \\
13 & & & & & & & \\
\end{array}
\]
Cleavage of AdoHcyase by SPE B

Fig 4
Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis

Hui-Ching Wang,1 Wenya Huang,2 Ming-Der Lai3 and Ih-Jen Su1,4,5

1Division of Clinical Research, National Health Research Institutes, Departments of 2Laboratory Science and Biotechnology, and 3Biochemistry, National Cheng Kung University College of Medicine, and 4Graduate Institutes of Basic Medicine, Biotechnology, and Clinical Medicine, National Cheng Kung University College of Medicine, Tainan, Taiwan

(Received March 7, 2006/Revised XXXX XX, 2006/Accepted March 28, 2006/Online Publication Month XX, XXXX)

Although hepatitis B virus (HBV) has been documented to cause hepatocellular carcinoma (HCC), the exact role of HBV in the development of HCC remains enigmatic. Several hypotheses have been proposed to explain the potential mechanism, including insertion mutagenesis of HBV genomes and transcriptional activators of HBV gene products such as HBx and truncated middle S mutants. In the past few years, we have identified two types of large HBV surface antigens (LHBs) with deletions at the pre-S1 (∆S1-LHBs) and pre-S2 (∆S2-LHBs) regions in ground glass hepatocytes. The pre-S mutant LHBs are retained in the endoplasmic reticulum (ER) and escape from immune attack. The pre-S mutants, particularly ∆S2-LHBs, are increasingly prevalent in patients with HBeAg-positive chronic HBV infection, ranging from 6% before the 3rd decade to 35% in the 6th decade. In HCC patients, the two pre-S mutant LHBs were detected in 60% of HCC patients, in the serum and in HCC tissues. Pre-S mutant LHBs can initiate ER stress to induce oxidative DNA damage and genomic instability. Furthermore, pre-S mutant LHBs can upregulate cyclooxygenase-2 and cyclin A to induce cell cycle progression and proliferation of hepatocytes. In transgenic mice, the pre-S mutants can induce dysplasia of hepatocytes and development of HCC. In a nested control study, the presence of pre-S mutants carried a high risk of developing HCC in HBV carriers. In summary, the findings in this review suggest a potential role for HBV pre-S mutants in HBV-related hepatocarcinogenesis, providing a model of viral carcinogenesis associated with ER stress. (Cancer Sci 2006)

Hepatitis B virus is recognized as a major etiological factor in the development of HCC.1,2 Epidemiological studies have demonstrated an approximately 100-fold increase in the relative risk of HCC among HBV carriers compared to non-carriers.3 Although the relationship between chronic HBV infection and HCC has been well established, the exact role of HBV in the pathogenesis of HBV-related hepatocarcinogenesis remains to be elucidated.

Hepatitis B virus is a partially double-stranded DNA virus containing a genome of 3.2 kb in size, which contains four open reading frames encoding viral polymerase, the core and e antigens, the HBx protein and the pre-S2/S gene encoding the three surface antigens (i.e., the large [pre-S1 + pre-S2 + S], middle [pre-S2 + S] and small [S only] surface proteins). In chronic HBV infection, HBV DNA can be integrated into the host genome. Almost all HCC harbor single or multiple copies of integrated HBV DNA.4,5 The integrated HBV DNA in tumors is usually rearranged and partially deleted. HBV DNA integration has been shown to be a random event and no specific cis-effect has been observed on flanking cellular genes.6,7 Therefore, HBV DNA integration per se is not considered to be a general mechanism of HBV-related hepatocarcinogenesis. Based on the observations obtained from several isolates of human HCC tissues, the integrated HBV DNA usually has truncated open reading frames coding for viral polymerase and the core antigen, and can only encode two gene products: the HBx and HBs proteins. Therefore, HBx and HBs proteins represent the two potential candidate proteins involved in HBV-related hepatocarcinogenesis. Although still controversial, the HBx protein has been studied extensively. It has been shown to exhibit transactivating functions, and to activate JAK/STAT and the Ras-Raf-MAPK signal pathway.8,9 In the case of HBs protein, the LHBs has been demonstrated to exert a tumor promoter-like function in the development of HCC.10,11 The C-terminally truncated middle surface protein MHBt has been recognized as a transactivator and initiates e-Raf-1/MAPK-2 signaling.12,13 In past years, we identified two types of LHBs with deletions at the pre-S1 (∆S1-LHBs) and pre-S2 (∆S2-LHBs) regions in GGH obtained from patients with advanced diseases of chronic HBV infection. These naturally occurring pre-S mutant LHBs are increasingly prevalent in serum and liver tissues of patients with chronic HBV hepatitis and in HCC tissues. The pre-S mutant LHBs are retained in the ER and induce ER stress signals. Furthermore, the pre-S mutant LHBs can upregulate COX-2 and cyclin A to induce cell cycle progression. In transgenic mice, ∆S2-LHBs has been shown to induce dysplastic changes in hepatocytes and development of HCC. In this review, we describe the biological

To whom correspondence should be addressed. E-mail: suijian@nhi.org.tw
Abbreviations: COX-2, cyclooxygenase-2; ER, endoplasmic reticulum; GGH, ground glass hepatocytes; HBsAg, HBs; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, XXX; MHBt, transactivating domain-binding protein t; LHBs, large surface protein; MHBt, nuclear factor NFkB; MAPK, ERK, retinoblastoma protein; S1-LHBs, LHBs with a deletion at the pre-S1 region; ∆S2-LHBs, LHBs with a deletion at the pre-S2 region; UPR, unfolded protein response.
characteristics of HBV pre-S mutants and their possible roles in the development of HCC based on the data mainly from our past studies.

Identification of pre-S (LHBs) mutants with deletions at the pre-S1 and pre-S2 regions in GGH

Ground glass hepatocytes have been recognized as the histopathologic hallmark of chronic HBV infection since 1973 when Hadziyannis et al. demonstrated the presence of HBV surface antigens in the 'glassy' cytoplasm of hepatocytes (Fig. 1a)\(^{[4,15]}\). Ultrastructurally, GGH are characterized by an abundance of ER in which HBsAg is accumulated.\(^{[16]}\) Several types of GGH have been recognized and correlated to the different replicative stages of chronic HBV infection.\(^{[17-20]}\) Of particular interest is the emergence of a so-called 'marginal type' GGH (type II GGH) at advanced stages of chronic HBV infection.\(^{[20]}\) Marginal type GGH harbor HBsAg at the cell margin or periphery of hepatocytes, distinct from the 'inclusion-like' accumulation of HBsAg in classic GGH (type I GGH; Fig. 1b). Furthermore, marginal type GGH consistently cluster in groups or nodules in liver tissues, suggesting a clonal proliferation or growth advantage of type II GGH.

The differential expression patterns of HBsAg in GGH imply the possibility of the existence of different types of HBV surface antigens. By laser-capture microdissection, we identified two major types of LHBs or pre-S mutant LHBs with deletions at either the pre-S1 (\(\Delta S1\)-LHBs) or pre-S2 (\(\Delta S2\)-LHBs) region in type I and type II GGH, respectively (Fig. 1c).\(^{[17,20]}\) This interesting finding drives us to explore in depth the potential biological significance of GGH, particularly the role of type II GGH or \(\Delta S2\)-LHBs in the pathogenesis of HBV-related tumorigenesis.

Pre-S mutants represent immune selection variants and are increasingly prevalent during the course of chronic HBV infection

The presence of different types of viral variants in different replicative stages of chronic HBV infection suggests the potential evolution of viral variants under immune pressure during HBV infection. Naturally occurring pre-S mutants are detected frequently in serum obtained from patients with chronic HBV infection.\(^{[24]}\) The resulting mutants reveal shorter forms of LHBs proteins with internal deletions. By examining serum samples obtained from longitudinal studies of patients with chronic hepatitis B over 10 years, we observed the sequential appearance of a wide spectrum of LHBs mutants, with the first appearance of variants with point mutations at the S region, followed by the presence of \(\Delta S1\)-LHBs, and later the emergence of \(\Delta S2\)-LHBs after \(\Delta S1\) conversion (W. C. Chen, I. J. Su et al., unpublished data).

The deletion site (nucleotides 4-57) of \(\Delta S2\)-LHBs has been recognized to correlate with the epitope of the CD8 T-cell response and B-cell neutralization.\(^{[25]}\) The pre-S2 mutant \(\Delta S2\)-LHBs may therefore represent an immune escape mutant that usually appears at the later stage of chronic HBV infection. In liver biopsy tissues of chronic HBV infection, there is usually an absence of lymphocyte infiltration in regions of type II GGH, supporting the concept of an immune escape for pre-S2 mutant \(\Delta S2\)-LHBs.

We have recently observed the prevalence of pre-S mutants in patients with HBsAg-positive chronic HBV infection who received antiviral therapy. The prevalence of pre-S mutants in serum increases from around 6% at the early stage (2nd decade of age) of chronic HBV infection with high HBV viral load, to the peak level of 35% during the 5–6th decade with low titers of HBV DNA. In patients with HCC, the prevalence rate of pre-S mutants increases up to 60% in both serum samples and liver tissues. Furthermore, the \(\Delta S2\)-LHBs appear to predominate over \(\Delta S1\)-LHBs in serum obtained from advanced diseases and in HCC tissues.
Pre-S mutant proteins are accumulated in the ER and induce ER stress signals

Using double-labeled immunofluorescence staining, we observed consistent colocalization of pre-S mutant proteins and the ER protein calnexin, suggesting that these pre-S mutant proteins, both the AS1-LHBs and AS2-LHBs, accumulate in the ER. Ultrastructurally, AS2-LHBs could induce a significant proliferation of the ER to an extent that is similar to the characteristics of GGH in the liver (Fig. 1d). The accumulation of pre-S mutants in ER may subsequently induce ER stress signals, leading to the induction of ER chaperones (GRP78 and GRP94). ER stress, also called the UPR in mammalian cells, is a cellular defense mechanism that responds to unfolded viral proteins or perturbed ER functions. Expression of viral gene products usually induces the UPR, which is sensed by two ER transmembrane kinases (IRE1 and PERK), and one ER transmembrane transcription factor (ATF6). These three UPR sensors are associated with ER chaperone GRP78/BIP when resting, and are dissociated from GRP78 upon ER stress. In our laboratory, we have demonstrated that the GRP78 gene is induced significantly in both types of GGH and in HCC, compared with non-LHBs-expressing hepatocytes. It has been reported that the induction of GRP78 may prevent cells from death by forming a complex with caspase-7 and caspase-12, and ER stress-regulated translation may increase tolerance to extreme hypoxia and promote tumor growth. The activation of ER-stress downstream molecules such as ATF-6, GRP78 and XBP-1 is believed to be involved in hepatocarcinogenesis. These data suggest that ER stress may play a contributing role in cell transformation, especially in HCC.

Pre-S mutant-induced ER stress may induce genomic instability through oxidative stress, increased DNA damage, impaired DNA repair and increased mutation frequency

The pre-S mutants may induce oxidative DNA damage through ER stress signaling pathways. Huh-7 cells carrying the pre-S mutant LHBs exhibited enhanced levels of reactive oxygen species and oxidative DNA damage. Furthermore, pre-S mutant LHBs can induce mutations on the X-linked lpr1 gene. Oxidative DNA damage could also be observed in livers of transgenic mice carrying pre-S mutant LHBs, as well as in GGH. Therefore, through ER stress signaling pathways, the pre-S mutant LHBs can induce oxidative stress and lead to oxidative DNA damage of HBV-infected hepatocytes. The oxidative DNA damage caused by pre-S mutant LHBs may result in genomic instability and mutation of liver cells, and ultimately lead to HCC.

Recently, preliminary studies from our group revealed that AS2-LHBs, but not wild-type or AS1-LHBs, could induce hyperphosphorylation of tumor suppressor RB. The AS2-LHBs could interact directly with JAB1, dissociating JAB1 from the JAB1-IRE1 complex in the ER lumen and causing JAB1 to translocate to cell nuclei. JAB1 is an important factor for modulating the level of cyclin-dependent kinase inhibitor p27Kip1, and targets nuclear p27Kip1 to the cytosolic 26S pro teaseosome for degradation. The degradation of p27Kip1 activates the phosphorylation of RB. Therefore, the combined events of oxidative DNA damage and RB hyperphosphorylation may represent two independent events for hepatocellular carcinogenesis associated with the ER viral protein AS2-LHBs.

Pre-S mutants may upregulate COX-2 through NfκB and p38 MAPK

Overexpression of COX-2 has been detected in many types of cancer and is linked to disease progression and survival of candidate cells. One of the many roles of pre-S mutan LHBs is to regulate the expression of COX-2. Constitutive or inducible expression of pre-S mutant LHBs induces the expression of COX-2 and prostaglandin E2 in immortalized mouse liver ML-1 cells and in transformed human Huh-7 cells. Transgenic mice expressing pre-S mutant LHBs express higher levels of COX-2 protein in liver and kidney tissues. Similarly, increased expression of COX-2 mRNA was observed in human HCC tissues expressing pre-S mutant LHBs. COX-2 induction is apparently important for the anchorage-independent growth conferred by the expression of pre-S mutant LHBs. Addition of etofolac, a specific COX-2 inhibitor, can abolish the growth of ML-1 cells expressing pre-S mutant LHBs in soft agar.

The induction of COX-2 is mediated mainly through transcriptional activation as actinomycin D (an inhibitor of RNA transcription) can attenuate the expression of COX-2 significantly. The transcription factor NF-κB is essential for transcription activation of the COX-2 promoter. Nuclear translocation of NF-κB, especially the p65 subunit, is observed when cells are treated with tunicamycin and brefeldin A, two ER stress inducers. NF-κB inhibitor can also block the induction of COX-2 by either an artificial ER stress inducer such as tunicamycin or the expression of pre-S mutant LHBs. Similar to our findings, NF-κB has been proposed to play a major role in the progression of inflammation-associated cancer. The activation of NF-κB is not only involved in the traditional degradation of IxBk but is also regulated by p38 MAPK. Inhibition of p38 MAPK does not affect the nuclear translocation of NF-κB but does inhibit NF-κB DNA binding activity and attenuate the induction of COX-2.

Taken together, the above-described role of NF-κB and COX-2 in the signal transduction of pre-S mutant LHBs suggests that they may act as potential preventive or therapeutic targets for HBV-related HCC. COX-2 inhibitor has been verified for the chemoprevention of many human cancers. The chemoprevention of liver cancer with COX-2 inhibitor may deserve future clinical trials.

Pre-S2 mutant AS2-LHBs can selectively upregulate cyclin A and induce cell cycle progression

Cyclin A is involved in both DNA synthesis and centrosome duplication during the cell cycle. It has been reported that both cyclin A and cyclin E are enhanced in HCC tissues and may be associated with tumor invasiveness and metastasis. Using cDNA microarray analysis, we observed that cyclin A, along with other cell cycle-regulated genes, was induced by AS2-LHBs. The induction of cyclin A was shown to be
initiated via the specific transactivator function of ΔS2-LHBs, independent of ER stress signals. The expression of ΔS2-LHBs in hepatocytes led to cell cycle progression under strong ER stress conditions and enhanced BrdU-incorporation with a multinucleation phenotype. Histopathological examinations revealed that cyclin A expression was enhanced in GGH, HCC tissues and transgenic mouse livers. One interesting finding is the tremendous expression of cyclin A in the cytoplasm of hepatocytes induced by ΔS2-LHBs. Although cyclin A functions predominantly in the nucleus, cytoplasmic expression of cyclin A may contribute to centrosome duplication. It is therefore interesting to clarify whether alteration of the subcellular localization of cyclin A could affect centrosome duplication that may subsequently contribute to cell aneuploidy. Recently, our study suggested that cytoplasmic localization of cyclin A is associated with its N-terminal truncation by a calcium-dependent protease, which is activated by ER stress (H. C. Wang and I. J. Su, unpublished data). These data suggest that cytoplasmic cyclin A may be initiated by ER stress and may contribute to aberrant centrosome duplication. The increased multinucleation and DNA aneuploidy observed in ΔS2-LHBs transgenic mouse livers supports the potential role of ER stress and impaired cyclin A during the development of HCC.

**Pre-S mutant LHBs can induce dysplastic changes and tumor formation in transgenic mice**

Hepatitis B virus transgenic mice have been produced both with complete HBV genomes that can support viral replication and with selected viral genes, including three surface proteins (large, middle and small HBs), HBe, core and precore proteins. Transgenic mouse studies have revealed a contributing role for HBe in hepatocarcinogenesis but the importance of HBx remains debatable. The overexpression of LHBs protein in transgenic mice has been shown to be cytopathic, and could lead to liver injury, regenerative hyperplasia, chronic inflammation, oxidative DNA damage, hepatocyte aneuploidy and eventually progression to HCC. In our laboratory, we demonstrated that ΔS2-LHBs can induce nodular proliferation and dysplasia of hepatocytes in transgenic mice, and tumor development was demonstrated recently (Fig. 2).

**Presence of pre-S mutants carries a high risk of developing HCC and represents a potential prophylactic target to prevent HCC development**

To test whether patients with HBV pre-S mutants carry a higher risk of HCC development, a case-control study nested in a cohort of 4155 HBsAg-seropositive residents was conducted to assess the risk of developing HCC in HBV carriers with or without pre-S mutants at enrolment. This nested case-control analysis involved 68 men with newly diagnosed HCC and 132 matched controls. Among these cases, 14 men with HCC (20.6%) and 11 controls (8.3%) had detectable pre-S mutants in serum samples at enrolment. After adjustment for age and HBeAg serostatus, the patients harboring pre-S mutants at enrolment had a higher risk of developing HCC than those without pre-S mutants during the 10-year follow-up period (OR = 3.2, 95% CI = 1.3–7.9, P = 0.01). These data indicate that the emergence of pre-S mutants may carry a high risk of developing HCC in patients with chronic HBV infection.

**HBV pre-S mutants may provide a model for viral carcinogenesis through latent ER stress signals**

The potential role of HBV pre-S mutants in the pathogenesis of HCC provides us with a model for viral carcinogenesis. The viral proteins may adopt a complex mechanism to escape from the host immune response by manipulating the machinery of cytoplasmic processing through the proteasome ubiquitination system. In this process, transport from the ER to the Golgi apparatus and cell membrane is manipulated meticulously by many viruses to escape from immune attack. In chronic HBV infection, a series of mutations under evolutionary CTL immune pressure may result in the emergence of pre-S mutants that accumulate in the ER. Retention of viral mutant proteins in the ER may induce ER stress and lead to apoptosis or, alternatively, the cells may try to survive through the activation of cell proliferation-related signals such as COX-2 and cyclin A to initiate cell proliferation (Fig. 3). In the presence of DNA damage and genomic instability, the proliferating cells may progress to tumor formation.

**Acknowledgments**

This research was supported by grants from the National Health Research Institutes and the Program to Promote the Excellence of Universities from the Department of Education, Taiwan (Dr Su).
Genetic polymorphisms in CYP1A1 and GSTM1 predispose humans to PCBs/PCDFs-induced skin lesions

Pei Chien Tsai a,b, Wenya Huang c, Yeu-Chin Lee a,
Shih Huang Chan d, Yueliang Leon Guo a,c,*

a Department of Occupational and Environment Health, National Cheng Kung University, Tainan 701, Taiwan, ROC
b Department of Basic Medical Sciences, National Cheng Kung University, Tainan 701, Taiwan, ROC
c Department of Medical Technology, National Cheng Kung University, Tainan 701, Taiwan, ROC
d Department of Statistics, National Cheng Kung University, Tainan 701, Taiwan, ROC
e Department of Environmental and Occupational Medicine, National Taiwan University Medical College, No. 1, Sec. 1, Jia-i Road, Taipei 100, Taiwan, ROC

Received 25 April 2005; received in revised form 3 August 2005; accepted 15 August 2005
Available online 3 April 2006

Abstract

Introduction: Polychlorinated biphenyls (PCBs) and dibenzo furans (PCDFs) are ubiquitous persistent pollutants in humans. Whether people with different genotypes are with different susceptibility to these chemicals are unknown. In a group of people highly exposed to PCBs/PCDFs, we tested the hypothesis that genotypic polymorphisms affected susceptibility for development of skin manifestations.

Methods: In 1979, approximately 2000 people in central Taiwan ingested cooking oil contaminated with PCBs/PCDFs. Skin disorder such as chloracne, abnormal nail, hyperkeratosis and skin allergy were found in PCBs/PCDFs exposed group. We recruited exposed and community background exposure subjects for blood testing and telephone-interview. Single nucleotide polymorphisms, AhR Arg554Lys, CYP1A1 Ile462Val, CYP1A1 T6235C, and GSTM1/T1 deletion, were determined. Occurrence of skin manifestations was compared among people with different genotypes while stratified by PCB exposure levels by logistic regression.

Results: Data on exposure, medical history, and genotypes were obtained from 393 exposed and 181 background exposure groups. Skin manifestations including chloracne, allergy, abnormal nail, and hyperkeratosis were more prevalent in exposed people in a dose-related manner. Among highly exposed individuals, combined CYP1A1-MspI mutant genotype and GSTM1-null genotype were associated with increased risk of chloracne (odds ratio 2.8, 95% confidence interval 1.1–7.6). Among intermediate-exposed individuals, GSTM1 null genotype was associated with skin allergy. AhR Arg554Lys genotype and GSTT1 null genotype were not related to susceptibility to skin manifestations in PCB/PCDF-exposed population.

Conclusion: CYP1A1 and GSTM1 genotypic polymorphisms might be related to the susceptibility to PCB/PCDF-induced skin manifestations.

© 2005 Elsevier Ltd. All rights reserved.

* Corresponding author. Address: Department of Environmental and Occupational Medicine, National Taiwan University Medical College, No. 1, Sec. 1, Jia-i Road, Taipei 100, Taiwan, ROC. Tel.: +886 922557708; fax: +886 6 274 3748.
E-mail addresses: pctsai@ms35.url.com.tw (P.C. Tsai), leonguo@mail.ncku.edu.tw (Y.L. Guo).

0021-9673/ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2005.08.012
1. Introduction

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants found in many environmental media and biological samples from human populations (Kimbrough, 1985; Jensen, 1987; Anderson, 1989). PCBs became recognized as potentially harmful environmental contaminants in the 1970s, and their use and manufacture were prohibited in many countries. Despite the ban on PCBs and cautious monitoring and control of dioxin-like chemicals, PCBs, PCDFs and PCDDs will be found in the environment many years to come due to their long half lives in the environment (Kimbrough, 1985). World Health Organization (WHO) has published recommendation on total daily intake of less than 1–4 pg toxic equivalency (TEQ)/kg body weight/day based on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Van Leeuwen et al., 2000). Although an uncertainty factor of 10 was taken into consideration, genotype-related individual susceptibility was not, probably due to relative lacking of detailed genetic susceptibility information.

Genetic polymorphisms might play some roles in human susceptibility to dioxin-like chemicals. Aryl hydrocarbon receptor (AhR) has been known as a ligand-activated receptor for TCDD and dioxin-like chemicals, such as coplanar PCB congeners and PCDFs (Reyes et al., 1992). The toxic potency of dioxin and PCB congeners is highly related to binding affinity to AhR. Three AhR genotype polymorphism sites belong to transactivation domain of exon 10 on chromosome 7, including codon 554 (Argnine to Lysine), 570 (Valine to Isoleucine) and 517 (Proline to Serine). AhR plays an important role to activate the transcription of xenobiotic metabolism as well as other genes (e.g., Cytochrome p450 family). In cultured human lymphocytes, combined Ile570 and Lys554 mutant genotypes were associated with higher TCDD-induced CYP1A1 expression, but such association was not shown on Lys554 mutant genotype only (Wong et al., 2001). In Asians, polymorphisms in codon 517 and 570 were rather rare.

The two most frequently reported CYP1A1 genotype polymorphic sites included thymine to cytosine (T to C) transition at position 6235, creating an additional cleavage site for MspI and a replacement of adenine by guanine (A to G) or isoleucine to valine (Ile to Val) at position 4889, which could be digested by NcoI restriction enzyme. The polymorphisms (T6235C and A4889G) mentioned above were found associated with the inter-individual differences in activity of arylhydrocarbon hydroxylase (AHH), which was one of the CYP1A1 enzymes (Landi et al., 1994; Persson et al., 1997). In animals and cultured human lymphocytes, CYP1A1 and 1B1 expression were induced by TCDD treatment (Vanden Heuvel et al., 1993; Spencer et al., 1999; Shimada et al., 2002).

Glutathione-S-transferase (GST) enzymes conjugate hydrophobic and electrophile compounds with reduced glutathione to detoxify these chemicals (Evans and Reling, 1999; Yuspa and Shields, 2001). Among the GST enzyme family, genetic polymorphisms on GSTM1 and GSTT1, involving deletion of 20 base pairs, respectively (Seidegard et al., 1986; Pembble et al., 1994), result in deficiencies in the enzyme activities. Accumulated evidences have suggested associations between GSTM1 null genotype and various cancers, including smoking-induced lung cancer, bladder cancer, colon cancer, and breast cancer (Alexandre et al., 1994; Anttila et al., 1994; d’Errico et al., 1996); and between GSTT1 null genotype and smoking-induced chromosome aberrations (el-Zein et al., 1997) and bladder cancer (Sorsa et al., 1996). Theses genetic polymorphisms are therefore considered important susceptibility markers for environmental toxicants.

In 1979, over 2000 people in central Taiwan ingested cooking oil contaminated with PCBs/PCDFs. The episode was later called Yucheng (“oil disease” in Chinese). The victims were estimated to consume an average of 1 g of PCBs and 3.8 mg of PCDFs totally during an average of nine months of exposure to the contaminated oil (Lin et al., 1981). Among their clinical manifestations, dermatological symptoms were most frequently reported, including chloracne, hyperkeratosis, abnormal nails, and skin allergy (Guo et al., 1999). These were similar to those found in the Japanese Yusho (“oil disease” in Japanese) population (Kuratsune et al., 1971; Nakayama et al., 1997).

In this study, we test the hypothesis that genetic polymorphisms of AhR, CYP1A1 and GSTM1/T1 are associated with skin manifestations in people highly exposed to PCBs/PCDFs.

2. Materials and methods

2.1. Study population

In 1993, 795 Yucheng subjects and 693 background exposure subject were telephone-interviewed for complete medical history, as detailed in the previous study (Guo et al., 1999). Yucheng subjects reported more chloracne, hyperkeratosis, abnormal nails, and skin
allergy than the background exposure groups. Among them, 405 Yucheng individuals had serum levels of PCBs analyzed in 1980–1982 by means of packed-column, electron-capture gas chromatography and an adaptation of the Webb–McCall method to a computer data system in which PCBs (Kanechlor 500) were used as reference standards. Background exposure subjects had mean serum PCB levels of 1.67 ppb wet weight as previously reported (Guo et al., 1997). We recruited these subjects for genotype determination between 2000 and 2003.

2.2. Genotype determination

DNA was isolated from peripheral blood lymphocyte by phenol–chloroform extraction. The polymerase chain reaction combined with restriction fragment length polymorphism (PCR/RFLP) was used for the genetic polymorphism analysis for AhR and CYP1A1. To detect AhR single nucleotide polymorphism, DNA (0.5 μg) samples were amplified in 50 μl reaction mixture containing 0.4 μM of each primer (Table 1), 1 U Taq DNA polymerase (AmpliTaq; Perkin Elmer) and 0.15 μM of dNTP (Boehringer Mannheim GmbH, Mannheim, Germany). The PCR reactions were performed with an initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplification products were digested with MseI at 37 °C for 3 h and then were analyzed on a 4% Nusieve 3:1 agarose gel (BMA, Rockland, ME USA). The amplification product contained a MseI site which can be detected in all individuals and was used as an internal control for the restriction enzyme activity. The 126 bp DNA fragment amplified from homozygous wild type was digested into two lengths: 92 bp and 34 bp (Arg/Arg) while the fragment from homozygous rare allele had three bands: 71 bp, 34 bp and 21 bp (Lys/Lys).

The CYP1A1 gene polymorphisms were separately amplified with the 2 pairs of primers (A and B) as shown in Table 1. PCR/RFLP profiles were based on the method of Hayashi et al. (1991) and Shields et al. (1993). For primers pair A, when an MspI restriction site was present, the 340 bp PCR product would be digested into two lengths: 140 bp and 200 bp. Homozygous wild type (m1/m1) lacked MspI cutting site. The 195 bp DNA fragment amplified with CYP1A1 primers pair B from homozygous wild type individual would be digested into two lengths: 163 bp and 32 bp (Ile/Ile) with NcoI. The homozygous rare allele (Val/Val) lacked the NcoI digestion site had the large parent bands.

Genetic polymorphism analysis for the GSTM1 and GSTT1 genes was determined simultaneously in a single assay using a multiplex PCR approach based on the method of Arand et al. (1996). The DNA sample was amplified with 3 pairs of primers as shown in Table 1. The PCR produced three DNA fragments of 215 bp (GSTM1), 350 bp (albumin) and 480 bp (GSTT1). In both GSTM1 and GSTT1 polymorphisms, gene deletion were responsible for the existence of null alleles. Individuals homozygous with respect to a given null allele lack the respective PCR amplified DNA fragment. Thus, Albumin was used as an internal control for the PCR efficiency.

2.3. Statistical analysis

We entered the data in JMP 5.0 software (SAS Company) for statistical analysis. Serum levels of PCBs collected in 1980–1982 were classified into intermediate and high levels by median of PCB exposure level (51 ppb). The prevalence of each of the four diseases

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCR assay primers, product sizes and RFLP enzymes sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Primers (5'→3')</td>
</tr>
<tr>
<td></td>
<td>Forward Reverse</td>
</tr>
<tr>
<td>AhR</td>
<td>GAA TCT TGG ACA TAC GTC AG</td>
</tr>
<tr>
<td></td>
<td>AGG CAT TGA TTT TGA AGA CATT</td>
</tr>
<tr>
<td>CYP1A1 PairA</td>
<td>GAG TAG AAG GGT GTA GCC GC</td>
</tr>
<tr>
<td></td>
<td>TAG GAG TCT TGT CTC ATG CC</td>
</tr>
<tr>
<td>CYP1A1 PairB</td>
<td>GAA CTG CCA CTT CAG CTG TCT</td>
</tr>
<tr>
<td></td>
<td>CCA GGA AGA CCA GCD CAC CCC AGC GGG GCA</td>
</tr>
<tr>
<td>GSTM1</td>
<td>GAA CTC CCT GAA AAG CTA AAG C</td>
</tr>
<tr>
<td></td>
<td>GTT GGG CTC AAA TAT ACG G TG G</td>
</tr>
<tr>
<td>GSTT1</td>
<td>TTC CTT ACT GGT CCT CAC ATC TC</td>
</tr>
<tr>
<td></td>
<td>TCA CCG GAT CAT GCC CAG GA</td>
</tr>
<tr>
<td>Albumin</td>
<td>GGC CTC TGC TAA CAA GTC CTA C</td>
</tr>
</tbody>
</table>
Table 2
Demographic and personal characteristics of Yucheng and background exposure subjects of 1993 in Taiwan

<table>
<thead>
<tr>
<th>Sex exposure</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yucheng (n = 176)</td>
<td>Background (n = 180)</td>
</tr>
<tr>
<td>Age (yr)a</td>
<td>50.8 ± 0.9</td>
<td>53.1 ± 1.2</td>
</tr>
<tr>
<td>30–39 (%)</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>40–49 (%)</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>50–59 (%)</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>&gt;60 (%)</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>Education (yr)a</td>
<td>6.9 ± 0.3</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>Ever smokedb</td>
<td>106 (60.0%)</td>
<td>67 (67.0%)</td>
</tr>
<tr>
<td>Ever drinkingb</td>
<td>93 (52.9%)</td>
<td>50 (50.0%)</td>
</tr>
<tr>
<td>PCB level (ppb)c</td>
<td>57.4</td>
<td>-</td>
</tr>
</tbody>
</table>

a Age and years of education were compared by unpaired t-test (mean ± standard).

b Age distribution, smoking history and consumption of alcoholic beverages were compound by chi-square test (values in parentheses are percent).

c PCB levels were measured by median of level in 1980-1982.

Fig. 2. Disease prevalence according to PCB levels in 1980-1982. PCB exposed group were divided into “intermediate”: PCB levels < 51 ppb and “high”: PCB level > 51 ppb. *P-value < 0.001* by Chi-square test in three groups.

(mean of PCB level: 29.0 ± 7.3 ppb); 168 highly exposed (mean of PCB level: 140.0 ± 7.4 ppb). Prevalence of skin diseases included chloracne, abnormal nail, hyperkeratosis and skin allergy were significantly increased in Yucheng groups, in a dose-response manner (Fig. 2).

3.3. Allele frequency in genetic polymorphisms

Five genetic polymorphisms included AhR-MspI, CYP1A1-MspI, CYP1A1-NcoI, GSTT1, and GSTM1 are shown in 393 Yucheng and 181 background exposure subjects (Table 3). The common allele frequency of AhR at coden 554 (Arg) in Yucheng group and background exposure group was 0.69 and 0.66, comparable to those among Japanese (0.57; n = 227) and Canadian Chinese (0.68; n = 41). Both polymorphisms of CYP1A1 at MspI and NcoI site had common allele frequencies of 0.57 and 0.75, respectively, in Yucheng population, and 0.60 and 0.77, respectively, in background exposure. GST-M1 null genotype and GST-T1 null genotype accounted for approximately a half of both Yucheng and background exposure groups.

3.4. Genetic susceptibility to diseases

In Yucheng population, logistic regression was done for each PCB exposure level stratum, which demonstrated that GST-M1 null genotype were associated with skin allergy only in the intermediate exposed group (OR = 3.1; 95% CI: 1.5–6.7). The other diseases were not associated with the five genetic polymorphisms. The results of single nucleotide polymorphism and diseases were shown in Table 4. Gene-gene interactive effects on skin diseases between three genetic polymorphisms (AhR, CYP1A1 MspI and GSTM1) were shown in Table 5. In people highly exposed to PCBs, the risk of chloracne was associated with combined CYP1A1 MspI mutant (m2/m2) and GSTM1 null genotype (OR = 2.8; 95% CI: 1.1–7.6). Among immediately exposed subjects, GSTM1 null genotype were related to skin allergy in both CYP1A1 genotypic groups (CYP1A1 wild/heterozygote, OR: 2.7; 95% CI: 1.2–6.1; CYP1A1 mutant, OR: 3.9; 95% CI: 1.2–12.6).

4. Discussion

This is the first study on genetic susceptibility to PCBs/PCDFs in highly exposed humans. The development of chloracne was more prominent in those with CYP1A1 MspI variant genotype (m2/m2) combined
### Table 3
Allele frequency of each genetic polymorphism in Yucheng and background exposure subjects

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Yucheng subjects (n = 393)</th>
<th>Background exposure (n = 181)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>AhR</td>
<td>Mscl</td>
<td>191</td>
<td>157</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>(Arg/Lys)</td>
<td>(0.49)</td>
<td>(0.40)</td>
<td>(0.11)</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Mspl</td>
<td>131</td>
<td>185</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>(C/T)</td>
<td>(0.33)</td>
<td>(0.47)</td>
<td>(0.20)</td>
</tr>
<tr>
<td></td>
<td>Ncol</td>
<td>226</td>
<td>139</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>(A/G)</td>
<td>(0.58)</td>
<td>(0.35)</td>
<td>(0.07)</td>
</tr>
<tr>
<td>GST</td>
<td>T1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>205</td>
<td>188</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.52)</td>
<td>(0.48)</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>M1</td>
<td>213</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.54)</td>
<td>(0.46)</td>
<td></td>
</tr>
</tbody>
</table>

W: wild genotype; H: heterozygous genotype; M: mutant genotype; Af: Allele frequency.
* Chi-square test comparing W, H, M genotype between Yucheng and background exposure groups.

### Table 4
The association of genetic polymorphism and diseases by PCB level in Yucheng group

<table>
<thead>
<tr>
<th>PCB level*</th>
<th>OR (95% CI)</th>
<th>Chloracne</th>
<th>Abnormal nail</th>
<th>Skin allergy</th>
<th>Hyperkeratosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (n = 168)</td>
<td>CYP1A1-Mspl (m2/m2 vs. m1/m1 or m2)</td>
<td>1.9 (0.8-4.2)</td>
<td>1.0 (0.4-2.4)</td>
<td>1.3 (0.6-2.9)</td>
<td>1.5 (0.4-4.7)</td>
</tr>
<tr>
<td>GMO-M1 (null vs. non-null)</td>
<td>1.5 (0.8-2.3)</td>
<td>2.3 (0.9-7.8)</td>
<td>1.2 (0.6-2.4)</td>
<td>1.3 (0.4-4.3)</td>
<td></td>
</tr>
<tr>
<td>GST-T1 (null vs. non-null)</td>
<td>1.2 (0.6-2.4)</td>
<td>1.5 (0.7-3.4)</td>
<td>0.8 (0.4-1.7)</td>
<td>1.1 (0.4-3.6)</td>
<td></td>
</tr>
<tr>
<td>AhR-Mscl (Lys/Lys vs. Arg/Arg or Lys)</td>
<td>2.0 (0.6-6.4)</td>
<td>1.3 (0.3-4.6)</td>
<td>0.4 (0.1-1.7)</td>
<td>0.9 (0.1-5.2)</td>
<td></td>
</tr>
<tr>
<td>Intermediate (n = 169)</td>
<td>CYP1A1-Mspl (m2/m2 vs. m1/m1 or m2)</td>
<td>0.9 (0.3-2.4)</td>
<td>-</td>
<td>1.2 (0.5-2.8)</td>
<td>-</td>
</tr>
<tr>
<td>GMO-M1 (null vs. non-null)</td>
<td>0.9 (0.1-3.8)</td>
<td>0.7 (0.0-4.2)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>GST-T1 (null vs. non-null)</td>
<td>0.6 (0.3-1.2)</td>
<td>0.6 (0.2-1.6)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AhR-Mscl (Lys/Lys vs. Arg/Arg or Lys)</td>
<td>1.0 (0.3-2.6)</td>
<td>0.3 (0.0-1.4)</td>
<td>0.8 (0.3-2.0)</td>
<td>1.3 (0.1-9.4)</td>
<td></td>
</tr>
</tbody>
</table>

(*) Means at least one genotype were zero.
* P-values < 0.05.
* PCBs level were classified intermediate and high by median level in 1980-1982.

Humans exposed to TCDD occupationally (Keetch et al., 1999) and environmentally (Caputo et al., 1988) were well known to develop chloracne. Chloracne is characterized by keratinized cutaneous cysts or comedones (Crow, 1970). Big cysts and comedones were often presented on below outer side of the eye, behind the ear, cheeks, forehead, auricles, trunk, inguinal region and with GST-M1 null genotype in highly exposed population. The other genotypes tested, namely, AhR, CYP1A1 Ncol site, GST-T1 null type were not associated with increased susceptibility of developing chloracne. In Yucheng subjects exposed to PCBs/PCDFs at intermediate levels, skin allergy was associated with GSTM1 null genotype.
Table 3
The interactive effects on skin problems among AhR, CYP1A1 and GSTM1 genotypes by PCB level in Yucheng group

<table>
<thead>
<tr>
<th>PCB level</th>
<th>PCB intermediate (n = 169)</th>
<th>PCB high (n = 168)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloracne</td>
<td>Skin allergy</td>
</tr>
<tr>
<td><strong>AhR</strong></td>
<td>GSTM1</td>
<td></td>
</tr>
<tr>
<td>WH</td>
<td>Non-null</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>Non-null</td>
<td>0.4 (0.1-1.7)</td>
</tr>
<tr>
<td>WH</td>
<td>Null</td>
<td>0.4 (0.2-1.0)</td>
</tr>
<tr>
<td>M</td>
<td>Null</td>
<td>1.6 (0.3-7.4)</td>
</tr>
<tr>
<td><strong>CYP1A1</strong></td>
<td>GSTM1</td>
<td></td>
</tr>
<tr>
<td>WH</td>
<td>Non-null</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>Non-null</td>
<td>0.7 (0.2-3.6)</td>
</tr>
<tr>
<td>WH</td>
<td>Null</td>
<td>0.5 (0.2-1.2)</td>
</tr>
<tr>
<td>M</td>
<td>Null</td>
<td>0.8 (0.2-6.0)</td>
</tr>
</tbody>
</table>

(-) Means that at least one of three dummy variables were zero.
* Means that P-value < 0.05.
* PCBs level were classified intermediate and high by median of level in 1980-1982.
+ CYP1A1 were digested by MspI restriction enzyme.
+ Logistic regression model were calculated with independent dummy variables.

external genitalia in PCBs/PCDFs exposure population (Kuratsune et al., 1995). Although the mechanisms
mediating chloracne development is not clear, one of
the hypotheses suggests that occurrence of chloracne
might be secondary to disturbance of Vitamin A (retinol)
homeostasis by TCDD, resulting in effects similar
to vitamin A deficiency (Nilsson and Hakansson,
2002). TCDD was reported to cause increased oxidation
and excretion of vitamin A, and reduced hepatic retinoid
levels (Nilsson et al., 2000). Oxidation of vitamin A
in turn induced acne formation. CYP1A1 has been
associated with TCDD-mediated oxidative stress (Nebert
et al., 1993), e.g., CYP1A1 was responsible for the
oxidation of 17β-estradiol to produce catechol estrogens.
It is possible that CYP1A1 activity is related to
TCDD-induced oxidation of vitamin A.

Genetic polymorphisms in human cytochrome P450
as well as phase II enzymes, such as GST, were related
to varied phenotypical expression of enzymes which can
influence metabolic activities and toxic effects.
CYP1A1 inducibility was shown to be greater in
CYP1A1 MspI variant genotype in mitogen-stimulated
lymphocytes (Landi et al., 1994), but such differential
induction was not associated with genetic polymorphism
at NcoI site (Persson et al., 1997). In addition, higher
CYP1A1 mRNA inducibility in response to 100 nM
TCDD was found in GSTM1 null genotype as compared
with non-null genotype in human lymphoblastoid
B cells (Vieary et al., 1993). In this study, increased sus-
cceptibility to developing chloracne in Yucheng subjects
with combination of GSTM1 null genotype and
CYP1A1 MspI variant genotype might have been due to
more readily induced hepatic enzymes, resulting in
more accelerated retinoid metabolism and excretion,
leading to abnormal sebocyte differentiation and hyper-
erkeratization of follicular canal, therefore the develop-
ment of acne (Paraskevaidis et al., 1998).

It was unclear whether glutathione-S-transferase
activity is related to TCDD toxicity. AhR and GST
activity were both found significantly depressed in the
peripheral lymphocytes among allergic and irritant con-
tact dermatitis subjects, as compared with control (Singh
et al., 1982). Whether such depression was related to null
genotype in GSTM1 is unknown. In Yucheng people,
GSTM1 genotype played an important role in suscepti-
bility to PCBs/PCDFs induced skin allergy, but only
among subjects with intermediate levels of exposure.
It is possible that in highly exposed subjects, exposure to
toxicants was sufficient to produce skin allergy disregard
to genotypes. Conversely, among individuals with inter-
mediate exposure, GSTM1 null genotype, thus a low-
ered capability of detoxifying these toxicants are also
required, in order to cause skin allergy.

In conclusion, we found that exposure to PCBs/
PCDFs causes skin lesions, including chloracne, skin
allergy, abnormal nail, etc. in a dose-related manner.
Genetic polymorphisms such as CYP1A1 and GSTM1
affected susceptibility to developing skin conditions
among the exposed individuals. Among those exposed
to intermediate levels of PCB/PCDFs, GSTM1 null
genotype predisposed people to developing skin allergy. Among Yucheng people, chloracne was associated with combined mutant CYP1A1-MspI and GSTM1 null genotypes. Although further studies are warranted to prove such findings since multiple comparisons could have caused false positive results, genotype variations and the related differences in susceptibility should be seriously considered while assessing risk in people exposed to PCBs and PCDFs, and related chemicals.

Acknowledgement

The study was supported by the National Science Council, Taiwan ROC NSC91-3112-B-006-006, and NSC93-3112-B-006-010.

References


among background, intermediate, and high exposure groups was compared by Chi-square test. The difference of allele frequencies in Yucheng and background exposure group was determined with Chi-square test for five genetic polymorphisms. We performed logistic regression analysis to determine relationship between single gene and each disease, as well as double genes and each disease, when controlled for exposure level. Odds ratio (OR) and 95% confidence intervals (CI) were calculated.

3. Results

3.1. Demographic characteristics

A completely telephone interview for 795 Yucheng subjects and 693 matched background exposure subjects were obtained, and the lifetime prevalence of chloracne, abnormal nails, hyperkeratosis, skin allergy, liver diseases and other symptoms in 1993 were recorded. Among them, 574 subjects (393 Yucheng and 181 background exposure) had genetic polymorphisms determined (Fig. 1). Table 2 compared the demographic and personal characteristics of Yucheng and background exposure groups. Stratified with sex, there is no significant difference in age, education level, smoking and consumption of alcoholic beverages in both groups. The median of PCB exposure levels in 1980–1982 (51 ppb) was far higher than that in pooled serum sample from general population (1.67 ppb) collected in 1992.

3.2. Dose-response relationship between PCB level and health effects

Among all subjects, 518 could be classified by PCB level in 1980–1982 into three groups, included 181 background exposure group; 169 with intermediate exposure
Glutathione S-transferase polymorphisms associated with risk of breast cancer in southern Taiwan

Tsai-Wang Chang\textsuperscript{a,b}, Shuei-Ming Wang\textsuperscript{c,1}, Yue-Liang L. Guo\textsuperscript{d}, Pei-Chien Tsai\textsuperscript{d}, Chien-Jung Huang\textsuperscript{e}, Wenya Huang\textsuperscript{c,*}

\textsuperscript{a}Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{b}Department of Surgery, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{c}Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{d}Department of Occupational and Environmental Health, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{e}Division of Endocrine and Metabolism, Department of Internal Medicine, Taipei City Hospital, Taiwan

Received 21 October 2005; received in revised form 8 March 2006; accepted 8 March 2006

KEYWORDS
Breast cancer; Glutathione S-transferase; BRCA1; Homologous recombination; DNA damage and repair.

Summary

In this study, the genetic polymorphisms associated with breast cancer in southern Taiwan were investigated. Two categories of genes were analyzed: (1) BRCA1, BRCA2, and Rad51, the DNA repair factors involved in homologous recombinational repair; and (2) CYP1A1, COMT, GST, and NAT2, the xenobiotic-metabolizing enzymes (XME) involved in estrogen metabolism. We found that the number of deletions and/or mutations in the GST genes was highly correlated with the occurrence of breast cancer. These data suggest that the GST enzymes, which detoxify the catechol estrogen quinones, are important target molecules for screening in populations at high risk of breast cancer.

© 2006 Elsevier Ltd. All rights reserved.

Introduction

Breast cancer occurs worldwide and greatly affects women’s health and social welfare. The onset of breast cancer is often earlier than other types of cancers, and often attacks women who play
important roles in families and professions. In Taiwan, the incidence of breast cancer has been increasing rapidly, probably due to the Westernization of life-style, especially eating habits. The peak of breast cancer onset in Taiwan is between 35 and 50 years of age, about 10 years earlier than in Western populations. More than 20% of breast cancers in Taiwan are diagnosed in women under 40 years of age. Therefore, screening for breast cancer in young women is an important issue. These observations suggest that Asian women are highly susceptible to breast cancer, and that the genetic background that may predispose young Taiwanese women to breast cancer needs to be investigated.

The risk of breast cancer is strongly associated with the level of estrogen exposure. Certain metabolites derived from estrogen induce genotoxicity and cytotoxicity. E2 (17 β-estradiol) has been reported to induce aneuploidy and chromosomal aberrations. E2 is hydroxylated by CYP enzymes and converted into reactive semiquinone derivatives. The catechol E2 semiquinone then undergoes redox cycling by cytochrome P450 oxidases and/or reductases and is converted into quinone derivatives that induce the formation of stable DNA adducts and initiate breast cancer. These reactive metabolites can alternatively be inactivated into harmless products by xenobiotic-metabolizing enzymes (XME) such as catechol-O-methyltransferase (COMT), superoxide dismutase (SOD), and a number of glutathione S-transferases (GST). Therefore, the activity of such detoxifying enzymes is important for protecting cells against DNA damage from catechol estrogen metabolites at each stage of metabolism.

Enzymes that regulate estrogen metabolites determine the exposure of breast cells to estrogen. Therefore, it is conceivable that the total level of activity contributed by the respective detoxifying enzymes is an important determinant of estrogen exposure in breast cells. Thus, the genotypes of various estrogen-metabolizing enzymes should be investigated together in order to understand the overall risks of breast cancer. Population study of the genetic polymorphisms in these respective genes is an essential step.

Mutations in the BRCA1 or BRCA2 genes, which are required for homologous recombinational repair (HRR), are often identified in cases of familial breast cancer. The HRR pathway is important in breast cancer, probably due to the high incidence of DNA strand breaks induced by estrogen and its metabolites in breast tissues. However, it is often difficult to assign a case of breast cancer as being either familial or sporadic due to the small numbers of siblings in contemporary families. A recent study by Hughes-Davies et al. reported that the ENSY protein, which is functionally linked to BRCA2, is associated with sporadic breast cancer, indicating the importance of screening for HHR genetic variations in populations of familial as well as sporadic breast cancers.

In order to determine the genetic risk factors associated with breast cancer in Taiwan, we examined the known genetic polymorphisms in genes involved in estrogen metabolism and homologous recombinational repair. Based on this study, the association of each estrogen-metabolizing enzyme with breast cancer risk is clarified and provides the basis for a strategy to screen for individuals genetically predisposed to breast cancer in Taiwan.

Materials and methods

Subjects

Peripheral blood was collected from 189 primary breast cancer patients and 421 age-matched females (healthy women who received a routine health examination and had no findings of cancer or any other medical problem) at the National Cheng Kung Medical Center, Tainan, Taiwan. All patients had been diagnosed histologically and had received breast resection surgery. All of the individuals received blood collection after obtaining informed consent. The response rates for cases and controls were both higher than 90%. Samples were stored at −80 °C until DNA extraction.

DNA Extraction

Genomic DNA was extracted from peripheral blood using standard procedures. Briefly, the blood was mixed with an equal volume of RBC lysis buffer (0.32 M sucrose, 5 mM MgCl2, 10% triton X-100, and 12 mM Tris–HCl, pH 8.0) then allowed to sit for 5 min. The cell lysates were then centrifuged at 2000 rpm and the upper layer was discarded. The pellets were re-suspended in phosphate buffered saline (PBS; pH 7.4) and 1% sodium dodecyl sulfate, and then digested with 0.2 mg/ml proteinase K at 55 °C overnight. After digestion, the DNA was purified using phenol-chloroform extraction, followed by ethanol precipitation with 0.3M potassium acetate (pH 5.2). The DNA was finally stored in TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) until use.
Genotyping

The gene variations tested and the primers used are listed in Table 1. The genes detected were amplified by the polymerase chain reaction (PCR). Briefly, each PCR mix contained 100 ng of genomic DNA with 25 mM N-[(Tris (hydroxymethyl) methyl)-3-aminopropanesulfonic acid (TAPS, pH 9.3), 50 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 200 μM of each dNTP, 0.25 μg/μl of activated calf thymus DNA, and two units of SuperTherm Fold DNA polymerase (JMAC Holdings, Kent, UK). The PCR reaction used the following sequence: 94°C for 10 min, then 35 cycles with each cycle consisting of 94°C for 60 s, 54°C for 45 s, and 72°C for 120 s. Finally, the PCR tubes were incubated at 72°C for 10 min. The DNA products were incubated with the appropriate restriction enzyme for 4 h, and then examined by agarose or polyacrylamide gel electrophoresis. In cases of BRCA1 intron 7 10-base

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Restriction Enzyme</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2</td>
<td>Forward 5'TGATCCATTTAGATTTCAATGGTACGCA-3'</td>
<td>NcoI</td>
<td>123</td>
</tr>
<tr>
<td>N372H</td>
<td>Reverse 5'GTTAAGATTCATTTCAATGGTACGCA-3'</td>
<td>HaeIII</td>
<td>104</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Forward 5'AAAACGCTCTGTGATTATTATT-3'</td>
<td>BspHI</td>
<td>62, 200, 338</td>
</tr>
<tr>
<td>M784V</td>
<td>Reverse 5'TAGCTCTGTGGAAGAGCAAAA-3'</td>
<td>VspI</td>
<td>400, 200</td>
</tr>
<tr>
<td>Rad51</td>
<td>Forward 5'TGGGAACTCACTCTACTCTCTTTG-3'</td>
<td>BstNI</td>
<td>86, 71</td>
</tr>
<tr>
<td>G153C</td>
<td>Reverse 5'GCCCTCTCTCTCTCTCACCACGG-3'</td>
<td>CII</td>
<td>157</td>
</tr>
<tr>
<td>Rad51</td>
<td>Forward 5'CCTGCGGATTTTATGACGACAGAC-3'</td>
<td>Styl</td>
<td>124</td>
</tr>
<tr>
<td>6174delT</td>
<td>Reverse 5'AATATTTGAAAAGACCGTCTGCA-3'</td>
<td>Del</td>
<td>102, 21</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Forward 5'TCAATATGCTATGACAATTTCTAG-3'</td>
<td>XbaI</td>
<td>23, 105</td>
</tr>
<tr>
<td>185delAG</td>
<td>Reverse 5'TCCCTCCATGATGTTAAGTTCAATTTC-3'</td>
<td>Del</td>
<td>126</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Forward 5'GCTGTAGTGTGCTATGACCAGATG-3'</td>
<td>N. A.</td>
<td>268</td>
</tr>
<tr>
<td>intron 7</td>
<td>Reverse 5'GAATCCAGCAATTATATTAT-3'</td>
<td>Del</td>
<td>258</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Forward 5'GATATTGTTAGTGACTAGACAGAG-3'</td>
<td>Hinfl</td>
<td>248, 67, 65</td>
</tr>
<tr>
<td>S382insC</td>
<td>Reverse 5'TTCGTGATCTCTTTAAAGTGCGCAGCT-3'</td>
<td>Ins</td>
<td>316, 65</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Forward 5'TAGGAGCTTTTCCTTCAAT-3'</td>
<td>Tsp50I</td>
<td>340</td>
</tr>
<tr>
<td>T6235C</td>
<td>Reverse 5'CGACAUAGAGGTTGACGACC-3'</td>
<td>MspI</td>
<td>200, 140</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Forward 5'GAGAGCTCATCTTACGT-3'</td>
<td>HindIII</td>
<td>139, 48</td>
</tr>
<tr>
<td>M62V</td>
<td>Reverse 5'GGAGAGCTCATCTTACGT-3'</td>
<td>VspI</td>
<td>120, 48, 19</td>
</tr>
<tr>
<td>COMT</td>
<td>Forward 5'AGGTGACGCTGAGCT-3'</td>
<td>NlaIII</td>
<td>136, 81</td>
</tr>
<tr>
<td>V158M</td>
<td>Reverse 5'AGGTGACGCTGAGCT-3'</td>
<td>MluI</td>
<td>96, 81, 40</td>
</tr>
<tr>
<td>NAT2</td>
<td>Forward 5'GGAAGAATTTGAGCTTTG-3'</td>
<td>KpnI, TaqI</td>
<td>706, 96, 93</td>
</tr>
<tr>
<td>Reverse 5'TCAGGATGAAATTAGCAG-3'</td>
<td>BamH1, MspI</td>
<td>362, 96, 32, 39</td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>Forward 5'GTAGTTTGGCCCCAGGTCAG-3'</td>
<td>BsrMI</td>
<td>329, 104</td>
</tr>
<tr>
<td>1105V</td>
<td>Reverse 5'AGGACCAAGGAGTTGTA-3'</td>
<td>VspI</td>
<td>222, 104</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Forward 5'GAACTCCTTGAAGCTAAGAC-3'</td>
<td>N. A.</td>
<td>215</td>
</tr>
<tr>
<td>Deletion</td>
<td>Reverse 5'GTGGAGCTCAATGATACGTGG-3'</td>
<td>N. A.</td>
<td>215</td>
</tr>
<tr>
<td>GSTT1</td>
<td>Forward 5'TCTCTTCTTCTACCTAC-3'</td>
<td>N. A.</td>
<td>480</td>
</tr>
<tr>
<td>Deletion</td>
<td>Reverse 5'CAACCTGAAAATGTTGCACTGCA-3'</td>
<td>N. A.</td>
<td>2768</td>
</tr>
</tbody>
</table>
deletion, the PCR products were analyzed with high-resolution agarose gel, which contained GelTwin agarose clarifier additive (J.T. Baker, Phillipsburg, NJ, USA) mixed with regular agarose in a 1:3 ratio.

Statistical analysis

The genotype distributions were analyzed to make sure that they were consistent with the Hardy–Weinberg equilibrium. JMP statistical packages (SAS) were used for the analysis. Logistic regression and multivariate logistic regression were used to examine the relationship between genetic polymorphisms and breast cancer. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Statistical significance was set at \( P < 0.05 \) based on a two-sided calculation. The following statistical analyses were carried out sequentially:

1. Univariate analyses compared the genotypic frequency of each polymorphism of the DNA repair and XME genes between cases and controls. Differences in frequency and the association between susceptibility genotypes and breast cancer risk were tested using logistic regression models, and the OR and the corresponding 95% CI for the association were estimated.

2. In this study, poor detoxification ability in the GST group was hypothesized to contribute to an increased risk of breast cancer, and women harboring putative high-risk alleles (the \( GSTP1 \) IV+VV alleles and the \( GSTM1 \) and \( GSTT1 \) deletion alleles) were considered to be at higher risk of cancer. Different combinations of these genotypes were compared with the low-risk genotypes (the \( GSTP1 \) II alleles and the \( GSTM1 \) and \( GSTT1 \) wild alleles).

3. Detoxification ability in the GST group was conceptualized as linear and additive. ORs were calculated for different total numbers of high-risk alleles (range 0–3).

4. Among people with lower numbers of high-risk GST genotypes and among people with higher numbers of high-risk GST genes, ORs were calculated for putatively high and low risk alleles of BRCA2, Rad51, CYP1A1, COMT, and NAT2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>No. (%)</th>
<th>Allele Frequency</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td><strong>DNA repair gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA2 N372H</td>
<td>NN+NH</td>
<td>394 (93.8)</td>
<td>176 (93.1)</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>HH</td>
<td>26 (6.2)</td>
<td>13 (6.9)</td>
<td>1.12 (0.55–2.19)</td>
</tr>
<tr>
<td>BRCA2 M784V</td>
<td>MM+MV</td>
<td>N.A.</td>
<td>86 (100)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>VV</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAD51 G135C</td>
<td>GG</td>
<td>284 (67.5)</td>
<td>116 (61.4)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>GC+CC</td>
<td>137 (32.5)</td>
<td>73 (38.6)</td>
<td>1.30 (0.91–1.66)</td>
</tr>
<tr>
<td><strong>XME gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1</td>
<td>TC+CC</td>
<td>287 (68.2)</td>
<td>118 (62.4)</td>
<td>0.57</td>
</tr>
<tr>
<td>T6235C</td>
<td>TT</td>
<td>134 (31.8)</td>
<td>71 (37.6)</td>
<td>1.29 (0.90–1.84)</td>
</tr>
<tr>
<td>CYP1A1 I462V</td>
<td>IV+VV</td>
<td>188 (44.7)</td>
<td>70 (37.3)</td>
<td>0.22</td>
</tr>
<tr>
<td>COMT V158M</td>
<td>VV+VM</td>
<td>391 (92.9)</td>
<td>180 (95.2)</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>30 (7.1)</td>
<td>9 (4.8)</td>
<td>0.64 (0.28–1.33)</td>
</tr>
<tr>
<td>GSTP1 I105V</td>
<td>II</td>
<td>288(68.4)</td>
<td>123(65.1)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>IV+VV</td>
<td>133(31.6)</td>
<td>66(34.9)</td>
<td>1.19 (0.82–1.71)</td>
</tr>
<tr>
<td>GSTM1 deletion</td>
<td>WT</td>
<td>193 (45.8)</td>
<td>82 (43.4)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Del</td>
<td>227 (54.2)</td>
<td>107 (56.6)</td>
<td>1.23 (0.79–1.57)</td>
</tr>
<tr>
<td>GSTT1 deletion</td>
<td>WT</td>
<td>210 (50.0)</td>
<td>78 (41.3)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Del</td>
<td>210 (50.0)</td>
<td>111 (58.7)</td>
<td>N.A.</td>
</tr>
<tr>
<td>NAT2</td>
<td>Fast</td>
<td>196 (78.4)</td>
<td>147 (77.8)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Slow</td>
<td>54 (21.6)</td>
<td>42 (22.2)</td>
<td></td>
</tr>
</tbody>
</table>

Total 421 189

The bold data demonstrate statistically significant differences between wild-type (WT) and mutants.
Results

Genetic polymorphisms associated with breast cancer

The genetic polymorphisms in DNA repair factors as well as estrogen metabolizing enzymes were examined. Allele frequencies in each gene detected (Table 2) were close to those reported in Caucasian and other Asian populations.16-19 Among the polymorphisms investigated, only the GSTT1 deletion had a statistically significant link with breast cancer, suggesting that the GSTT1 enzyme might play a critical role in the detoxification of quinones and protection against oxidative damage in cells.

We detected the known SNPs and mutations in the BRCA1, BRCA2, and RAD51 genes that had been reported to be associated with familial breast cancer in western countries.20-24 We found that BRCA2 N372H and RAD51 G135C, variations correlated with breast cancer risk in western countries, were not significantly higher in our breast cancer patients than in the controls (data not shown). Furthermore, the BRCA1/2 hotspot mutations in Ashkenazi Jewish familial breast cancers, BRCA1 185delAG and 5382insC, and BRCA2 6174delT, were not identified in any of our breast cancer patients.25,26 Recent studies27,28 reported that a 10-base deletion in BRCA1 intron 7 was identified in two Taiwanese families with breast cancer. Surprisingly, no such mutation was found in any of our breast cancer patients (data not shown).

Association of glutathione S-transferase polymorphisms with breast cancer

Polymorphisms in the GSTT1, GSTM1, and GSTP1 enzymes were sought in order to detect any association of these GST enzymes with breast cancer incidence. Fewer than 15% of our patients and controls had wild-type genotypes in all three of the GST genes (Table 3). The majority had at least one mutation, suggesting that most people do not exhibit full GST activity, but display individually variable levels of GST catalysis. Our analysis indicated that the risk for breast cancer was not significantly elevated unless all three GST genes carried mutations (Table 3). The OR of breast cancer risk in this group was as high as 3.49 times (CI = 1.41-9.22) higher than members of the other groups with wild-type genotypes in all three GST genes. The percentage of individuals with three mutations accounted for 13.8% of our breast cancer patients, indicating that mutation in these three GST genes is not rare.

To understand whether the combinations of mutations in GST and the other estrogen-metabolizing enzymes would greatly increase the risk of breast cancer, SNPs in the CYP1A1, COMT, and NAT2 genes were also examined. Our results indicate that when all three GST enzymes were mutated, any additional mutation in the CYP1A1 or NAT2 genes dramatically increased the risk of breast cancer (OR = 3.78-4.74, Table 4). Furthermore, a combination of mutations in the three GST enzymes along with the BRCA2 N372H or RAD51 G135C SNPs greatly increased the disease risk (OR = 2.94 and 2.67, respectively).

Discussion

In our examination of the association of breast cancer with polymorphisms in estrogen metabolites and DNA repair enzymes, we found that the total GST level, shown as variations in the GSTT1,

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Polymorphisms in GSTT1; GSTM1 and GSTP1 genes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST genes</td>
<td>No. (%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (W)+M1 (W)</td>
<td>53 (12.7%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (D)+M1 (W)</td>
<td>74 (17.7%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (W)+M1 (D)</td>
<td>84 (20.1%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (D)+M1 (D)</td>
<td>76 (18.2%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (W)+M1 (W)</td>
<td>29 (7.0%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (D)+M1 (W)</td>
<td>35 (8.4%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (W)+M1 (D)</td>
<td>12 (3.0%)</td>
</tr>
<tr>
<td>P1 (W)+T1 (D)+M1 (D)</td>
<td>24 (5.8%)</td>
</tr>
</tbody>
</table>

The bold data demonstrate statistically significant differences between wild-type (WT) and mutants.

W: wild-type, which contains one or two wild-type alleles in each gene: either homozygous or heterozygous.
D: Both GSTP1 alleles are variant (PV).
M: deletion detected and null phenotypes present in GSTM1 or GSTT1 gene.

The associations of breast cancer with these polymorphisms are consistent with previous findings.
Table 4  Combined genetic polymorphisms in GST and other genes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GST mutation</th>
<th>No. of case</th>
<th>OR* (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2 N372H (NH/HH)</td>
<td>0–2</td>
<td>83</td>
<td>1.18 (0.36)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>2.94 (0.0034)</td>
</tr>
<tr>
<td>RAD51 G135C (GC+CC)</td>
<td>0–2</td>
<td>63</td>
<td>1.33 (0.14)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>2.67 (0.034)</td>
</tr>
<tr>
<td>CYP1A1 T6235C (TT)</td>
<td>0–2</td>
<td>62</td>
<td>1.29 (0.19)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>4.74 (0.0063)</td>
</tr>
<tr>
<td>CYP1A1 H462V (II)</td>
<td>0–2</td>
<td>99</td>
<td>1.27 (0.20)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19</td>
<td>4.42 (0.00020)</td>
</tr>
<tr>
<td>COMT V158M (MM)</td>
<td>0–2</td>
<td>8</td>
<td>0.93 (0.70)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>2.85 (0.0079)</td>
</tr>
<tr>
<td>NAT2 (slow type)</td>
<td>0–2</td>
<td>31</td>
<td>0.89 (0.67)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11</td>
<td>3.78 (0.026)</td>
</tr>
</tbody>
</table>

The bold data demonstrate significantly enhanced odds ratios in the GST deletion (3) genotype compared with those in the GST deletion (0–2) genotype when the other genes have high-risk genotypes.

*Odds ratios are presented as comparisons between the GST deletion genotype (0–2) and the wild type of the other genes tested.

GSTM1, and GSTP1 genes, was critical for the risk of breast cancer. Mutations in all three GST genes resulted in oxidative stress and the retention of reactive quinone intermediates in cells. This cellular stress, resulting from defects in GST enzymes, was even more dramatic when other XME genes, such as CYP1A1, COMT, or NAT2, were also mutated. Therefore, individuals carrying mutations in all three GST genes are the population at highest risk of breast cancer. Approximately 8.2% of our cohort (breast cancer patients and controls) carried mutations in all of these genes. Therefore, screening for GST haplotypes is essential for cancer prevention.

The allele frequencies we found in XME genes were similar to those identified elsewhere. However, the mutational hotspots in the BRCA1 and BRCA2 genes identified in these studies were not found in our cohort, suggesting that BRCA1/BRCA2 mutations in southern Taiwan, if any, are quite distinct from those in western countries. Nevertheless, such mutations may have been missed in our study, due to the sample size. A previous study in Taiwan reported a 10-base deletion in BRCA1 intron 7 in a few familial breast cancer cases. This mutation was not found in any of our participants, however, indicating that the mutational hotspots associated with a familial predisposition to breast cancer in Taiwan have not yet been identified. This finding again suggests that the genetic polymorphisms in different ethnic groups or areas in Taiwan are quite distinct and that the findings in one group may not apply to others.

In cases of familial breast cancer, mutations in the BRCA1 and BRCA2 genes have been found to play important roles. Both BRCA1 and BRCA2 are involved in the DNA recombinational repair pathway, which detects and repairs DNA-strand breaks caused by chemical or physical agents. Estradiol (estradiol) has been reported to induce DNA strand breaks by intercalating with the DNA helix. Catechol estrogen metabolites also induce oxidative stress, resulting in guanine/adenine oxidation and DNA breakage. These DNA lesions are mainly repaired via the homologous recombinational repair pathway, which involves BRCA1, BRCA2, and RAD51. Tumor tissues from familial breast cancer patients are defective in DNA repair. In addition to its effect on DNA damage, which leads cells to genomic instability, estrogen also increases breast cell proliferation and mitosis, thereby increasing the possibility of mutation. This is another effect of estrogen on the initiation and progression of tumors in the breast. Taking these findings together with ours, we suggest that prolonged exposure to estrogen and to its decelerated metabolism is a major risk factor for breast cancer. Defects in DNA repair for estrogen adducts and estrogen derivatives are also significant risk factors for breast cancer, even the sporadic types.

In addition to XME factors, estrogen receptors (ER) and progesterone receptors (PgR) contribute to an individual's exposure to estrogen. It is well established that the genetic polymorphisms in estrogen receptors are associated with the risk of breast cancer. A recent study identified the ER
haplotypes associated with decreased or increased risk of breast cancer. Therefore, the investigation and genotyping of estrogen and progesterone receptors in humans appear to be crucial.

Considerable inter-individual variability has been observed in carcinogen metabolism as well as in the biosynthetic pathways and metabolism of steroid hormones. Given that XME factors are involved in the metabolism of and exposure to various carcinogens, they may help define the subpopulations with greater lifetime exposure to breast or other types of cancer. Although the XME genes have been considered to have low penetrance, acting together with endogenous and lifestyle risk factors, they are likely to account for a higher proportion (8.2% with variations in the GSTM1, P1, and T1 genes) of breast cancer cases, compared with high-penetrance genes such as BRCA1 and BRCA2, which contribute primarily to familial breast cancer.

In conclusion, our investigation of the genetic polymorphisms associated with breast cancer in southern Taiwan demonstrated that variations in the xenobiotic metabolizing enzymes and in the BRCA1/2 factors have synergistic effects on the incidence of breast cancer. This is the first study that classifies cancer risks by combinatorial analysis of a large number of different genetic polymorphisms. We recommend that our findings be kept in mind when screening subpopulations with a predisposition to breast cancer.

References

Preparation of Amino-acid-type Selective Isotope Labeling of Protein Expressed
in Pichia pastoris

Chiu-Yueh Chen¹, Chun-Ho Cheng¹, Yi-Chun Chen¹, Jenq-Chang Lee², Shan-Ho
Chou³, Wenyu Huang⁴, and Woei-Jer Chuang¹*

¹Department of Biochemistry and Institute of Basic Medical Science, National Cheng
Kung University College of Medicine, Tainan 701, Taiwan
²Department of Surgery, National Cheng Kung University College of Medicine,
Tainan 701, Taiwan
³Department of Biochemistry, National Chung Hsing University, Taichung 402,
Taiwan
⁴Department of Medical Technology, National Cheng Kung University College of
Medicine, Tainan 701, Taiwan

*Correspondence to Woei-Jer Chuang, Department of Biochemistry, National Cheng
Kung University College of Medicine, Tainan 701, Taiwan
Phone: 886-6-235-3535 ext. 5515; Fax: 886-6-274-1694; E-mail: wjcnmr@mail.ncku.edu.tw

Short Title: AATS isotope labeling of protein expressed in P. pastoris

Key words: amino-acid-type selective isotope; highly disulfide-bonded protein;
rhodostomin; NMR; Pichia pastoris; selective label; structure determination; X-ray

Grant sponsor: National Science Council of ROC, Grant numbers:
NSC-93-2311-B-006-004 and NSC-93-2323-B-006-001; Grant sponsor: Program for
Promoting University Academic Excellence, Grant number: 91-B-FA05-1-4.
Abbreviations

Abbreviations: AATS, amino-acid-type selective; Den, dendroaspin; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; P. pastoris, Pichia pastoris; Rho, rhodostomin.
ABSTRACT

We report the culture conditions for successful amino-acid-type selective (AATS) isotope labeling of protein expressed in Pichia pastoris (P. pastoris). Rhodostomin (Rho), a six disulfide-bonded protein expressed in P. pastoris with the correct fold, was used to optimize the culture conditions. The concentrations of [α-15N] selective amino acid, non-labeled amino acids, and ammonium chloride, as well as induction time, were optimized to avoid scrambling and to increase the incorporation rate and protein yield. The optimized protocol was successfully applied to produce AATS isotope-labeled Rho. The labeling of [α-15N]-Cys has ~45-55% incorporation rate, and all 12 cysteine resonances were observed in HSQC spectrum. The labeling of [α-15N]-Leu, -Lys, and -Met amino acids has an incorporation rate greater than 45%, and the expected number of resonances in the HSQC spectra were observed. In contrast, the labeling of [α-15N]-Asp and -Gly amino acids has a low incorporation rate and the scrambling problem. In addition, the culture condition was successfully applied to label dendroaspin (Den), a four disulfide-bonded protein expressed in P. pastoris. Therefore, the described condition should be generally applicable to other proteins produced in the P. pastoris expression system. This is the first report to present a protocol for AATS isotope labeling of protein expressed in P. pastoris for NMR study.
INTRODUCTION

One of the key steps of structural genomics and proteomics is to express target proteins. Many protein expression systems, including E. coli, P. pastoris, Baculovirus-infected insect cell, mammalian cell, and cell-free protein synthesis, have been established to produce recombinant proteins. Protein labeling is critical for successful structure determination of proteins using NMR spectroscopy and X-ray crystallography. A variety of methods are available to produce isotopically labeled proteins in protein expression systems, including methods for uniform labeling, backbone-only labeling, methyl-group labeling, amino-acid-type selective (AATS) labeling, segmental labeling, and reverse labeling.

The introduction of AATS isotope labeling of protein can aid in assigning the NMR resonances of large proteins and in obtaining the phase information of X-ray crystallography. AATS isotope-labeled proteins are used not only to simplify the process of NMR assignment but also to isolate spectral information from the region of interest. Even with triple-labeled proteins, it is difficult to interpret NMR structures of proteins larger than 40 kDa using current methods. One way to overcome this limitation is to label proteins selectively at certain amino acids. Selective labeling makes it possible to obtain structural information about particular amino acids in proteins larger than 40 kDa. The protocols of AATS isotope labeling have been developed for the protein expression systems of E. coli, Baculovirus-infected insect cell, mammalian cell, and cell-free protein synthesis.

To date, there is no culture condition to produce AATS isotope labeling of protein expressed in P. pastoris for NMR study.

The most common system used for protein labeling is the recombinant expression of proteins in E. coli in the presence of labeled nutrients or supplements.
However, many proteins cannot be expressed in *E. coli* with the correct fold.\textsuperscript{19} Therefore eukaryotic expression systems such as yeast, insect, or mammalian cells are used to overcome this problem.\textsuperscript{14} Even though these eukaryotic expression systems may allow us to express proteins with the correct fold, they have certain disadvantages. For example, most proteins obtained from these expression systems are expensive, time consuming, and low-yield.\textsuperscript{20} Recently the methylotrophic yeast *P. pastoris* has drawn a lot of attention because it combines several advantages of prokaryotic and eukaryotic expression systems.\textsuperscript{21-25} Many reports have shown that *P. pastoris* was developed into a highly successful system for the production of a variety of heterologous proteins. It is the only system that offers the benefits of *E. coli* (high-level expression, easy scale-up, and inexpensive growth medium) combined with the advantages of expression in a eukaryotic system (protein processing, folding, and post-translational modifications).\textsuperscript{23} The *P. pastoris* expression system has demonstrated a capacity for performing many post-translational modifications such as glycosylation, proteolytic processing, and disulfide bond formation.\textsuperscript{20-25} Specifically, this system has been successfully used to produce highly disulfide-bonded proteins.\textsuperscript{19,26} The advantage of the *P. pastoris* expression system is that it produces high-level expression of the gene of interest.\textsuperscript{24} *P. pastoris* can be easily grown to high cell densities using defined minimal media to produce high yields of proteins.\textsuperscript{24} Typically, the level of expressed protein that can be achieved using this expression system is as high as 30\% of the total cell protein. One of the key steps of protein structure determination is to introduce a stable isotope into proteins.\textsuperscript{1-3} The protocols to produce $^2$H, $^{13}$C, and $^{15}$N-labelled proteins in *P. pastoris* have been established.\textsuperscript{27-33} However, there is no protocol to produce AATS isotope labeling of protein expressed in *P. pastoris* for NMR study.
In order to develop the protocol to produce AATS-labeled protein in \textit{P. pastoris} expression system for NMR study, we need a protein expressed in \textit{P. pastoris} to optimize the culture condition. In a previous study we expressed a six disulfide-bonded protein, rhodostomin, in \textit{P. pastoris} with the correct fold.\textsuperscript{26,34} Rhodostomin is a disintegrin which inhibits platelet aggregation by blocking the binding of fibrinogen to the integrin \( \alpha_{\text{IIb}}\beta_{3} \) of platelets. We also showed that rhodostomin expressed in \textit{P. pastoris} possesses the same function and structure as those of native protein. Therefore, we used rhodostomin as the model protein to obtain the culture condition. After we obtained the culture condition, we also used recombinant dendroaspin (Den), a four disulfide-bonded protein expressed in \textit{P. pastoris}, to test whether the obtained culture condition can be applied to label other proteins expressed in \textit{P. pastoris}.\textsuperscript{35,36} In the present study, we were able to overcome the scrambling and low-yield problems and to disclose the AATS labeling protocol to produce proteins expressed in \textit{P. pastoris} with a 50-80% incorporation rate and protein yields of 9.5-13.4 mg/L.
MATERIALS AND METHODS

Expression and Purification of Rho and Dendroaspin

The expression and purification of Rho in *P. pastoris* was done following the protocols as described previously.\textsuperscript{26} We also expressed Den in *P. pastoris* as follows: A synthetic Den gene was constructed by ligating four complementary and overlapping oligonucleotides coded for the protein sequence of Den using *P. pastoris* codon data. The expression of Den in *P. pastoris* was done using yeast transfer vector (pPICZα A; Invitrogen Corp., Carlsbad, CA) and an expression kit (EasySelect version A; Invitrogen) according to the manufacturer’s instructions with minor modifications. The structural gene of Den was amplified using a polymerase chain reaction (PCR) with the sense primer: 5’-AATTCTGAATTCCATCATCATCATCATCATGGTTAAGGAATGTGACTGTCTTTT-3’ that has *Eco Rl* recognition and six histidine residues for facilitating purification. The antisense primer was: 5’-CCGCGGGCCGCTACGTGTATTTGACAGTCAGCAGCAG-3’ with *Sac II* recognition and a TCA stop codon. The PCR product was purified and then cloned into the *Eco Rl* and *Sac II* sites of pPICZαA. The recombinant plasmid was transformed into the DH5α strain, and the colony was selected using an agar plate with low salt LB (1% tryptone + 0.5% yeast extract + 0.5% NaCl + 1.5% agar; pH 7.0) and 25 μg/ml of the antibiotic phleomycin D1 (Zeocin; Invitrogen). After the clone was confirmed by sequencing the insert, a total of 10 μg plasmid was digested with *Sac I* to linearize the plasmid. The linearized construct was transformed into the *Pichia* strain X33 using the heat shock method, and the transformation was performed with a kit (*Pichia EasyComp*; Invitrogen). The transformant integrated at the 5’-AOX1 locus using single crossover, and the colony was selected using an agar plate with yeast peptone dextrose (YPD) (1% yeast extract, 2% peptone, 2% glucose,
and 2% agar) and 100 µg/ml Zeocin. We picked the highest Den protein expression clone from a number of clones with multi copies of the Den gene insertion.

We produced $^{15}$N-labelled Rho and Den as follows: 100 µl of cell stock was grown at 30°C in 200 ml of $^{15}$N minimal medium (0.34% yeast nitrogen base (YNB) without ammonium sulfate and amino acids, 2% dextrose, $4 \times 10^{-5}$% biotin, and 0.05% $^{15}$NH$_4$Cl) in 100 mM potassium phosphate buffer at pH 6 with 100 µg/ml Zeocin for 48 h. The cells were then transferred into 800 ml of $^{15}$N minimal medium. After another 48 h, the cell densities reached OD$_{600}$ of 25-30. The cells were then centrifuged, collected, and grown in 1 liter of $^{15}$N minimal methanol medium (0.34% YNB without ammonium sulfate and amino acids, $4 \times 10^{-5}$% biotin, 1% Methanol, and 0.05% $^{15}$NH$_4$Cl). Once every 24 h, 1% methanol was added to induce protein expression for 2 days. The supernatant was then collected using centrifugation for protein purification.

Selective [$\alpha$-$^{15}$N]-Ala-, -Asp-, -Cys-, -Gly-, -Lys-, -Leu-, and -Met-labeled proteins were produced as follows: 500 µl of cell stock was grown at 30°C in 200 ml of YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) containing 100 µg/ml Zeocin for 48 h. The cell culture was then transferred into 1300 ml of YPD medium. After growing for another 48 h, the cells obtained from 1.5 liters of culture were centrifuged, collected, and grown in 990 ml of a minimal medium described by LeMaster and Richards,¹⁸ but with some modification. The cell suspension was concentrated to an OD$_{600}$ of 25-30. The modified medium contained unlabeled L-amino acids (0.4 g asparagine, 0.5 g alanine, 0.4g arginine, 0.4 g aspartic acid, 0.05 g cysteine, 0.4 g glutamine, 0.65 g glutamic acid, 0.55 g glycine, 0.1 g histidine, 0.23 g isoleucine, 0.23 g leucine, 0.42g lysine hydrochloride, 0.25 g methionine, 0.13 g
phenylalanine, 0.1 g proline, 2.1 g serine, 0.23 g threonine, 0.17 g tyrosine, and 0.23 g valine), as well as 0.5 g adenine, 0.65 g guanosine, 0.2 g thymine, 0.5 g uracil, 0.2 g cytosine, 1.5 g sodium acetate, 0.5 g succinic acid, 0.25 g NH₄Cl, 0.85 g NaOH, and 10.5 g K₂HPO₄, and 10 ml of a filter-sterilized solution containing 25 mg MgSO₄.7H₂O, 28 mg FeCl₃.6H₂O, 2 mg CaCl₂.2H₂O, 2 mg ZnSO₄.7H₂O, 2 mg MnSO₄.H₂O, 50 mg L-tryptophan, 50 mg thiamine (vitamin B₁), 50 mg nicotinic acid, and 4 × 10⁻⁵% biotin; 5-100 mg of selective [α-¹⁵N]-labeled amino acid was substituted for the unlabeled amino acid in the medium. In the case of selenomethionine labeling, 90 mg was added to the medium. The medium was close to pH 7.2. A total of 1% methanol was added to induce protein expression for 24-48 h.

The culture supernatant was collected using centrifugation and dialyzed twice against 10 liters of H₂O and twice against 5 liters of binding buffer. The final solution was loaded into a nickel-chelating column, and the rhodostomin protein was eluted with a gradient of 200 mM imidazole. The recombinant proteins produced in P. pastoris were further purified using reverse-phase C18 high performance liquid chromatography (HPLC) with a gradient of 15% to 18% acetonitrile. The tricine SDS-PAGE analysis indicated that the purity of the proteins was greater than 95%.

Platelet Aggregation Assay

Venous blood (9 parts) from healthy donors who had not received any medication for at least two weeks was collected in 3.8% sodium citrate (1 part). Blood was centrifuged at 150 × g for 10 min to obtain platelet-rich plasma (PRP) and allowed to stand for 5 min, after which PRP was collected. Platelet-poor plasma (PPP) was prepared from the remaining blood by centrifuging it at 2000 × g for 25 min. The PPP platelet count was measured on a hematology analyzer and diluted to 250,000
platelets/µl. A solution of 190 µl of PRP and 10 µl of either Rho or PBS buffer was incubated for 5 min using an aggregometer (Hema Tracer 601; Nikoh Bioscience, Tokyo, Japan) at 37°C. Ten microliters of 200 mM ADP was added to monitor the response of platelet aggregation using light transmission.26

Mass Spectrometric Measurement

The molecular weights of AATS isotope-labeled proteins were examined using a triple quadrupole mass spectrometer (API365; PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). The proteins were dissolved in a matrix of 0.1% formic acid and 100% methanol. Incorporation rates of the [α-15N]-labeled amino acids into Rho were calculated using the ratio of relative abundances between the expected labeled mass peaks and the total of unlabeled and labeled mass peaks.

NMR Spectroscopy

NMR experiments were performed at 27 °C on a Bruker Avance 600 spectrometer equipped with pulse field gradients and xyz-gradient triple resonance probes at National Cheng Kung University. NMR samples were dissolved in 10% D2O at a concentration of 0.5-1 mM, and the pH values were adjusted with 100 mM KOD to 6.0. Experiments of 2D 15N-1H heteronuclear single quantum coherence (HSQC) and 1D 15N-edited and -filtered spin echo were used to determine the assignments of the labeled amino acid resonances and the incorporation rates of the 15N-labeled amino acids into protein, respectively.38 NMR spectra were processed and analyzed using the XWINNMR and Aurelia programs on an O2 Silicon Graphics workstation. The center frequencies of double-resonance experiments were 4.75 ppm (1H) and 118 ppm (15N). The observed 1H chemical shifts were referenced with respect to an H2O or HOD signal, which was taken as 4.754 ppm downfield from external sodium 3-trimethylsilylpropionate -2,2,3,3-d4 (TSP) in D2O (0.0 ppm) at 27
°C. The nitrogen chemical shift was referenced to external $^{15}$NH$_4$Cl (3 mM in 1M HCl) at 27 °C, which is at 24.93 ppm downfield from liquid NH$_3$. 
RESULTS

Expression, Purification, and Characterization of Rho Expressed in \textit{P. pastoris} 

To obtain a protocol for AATS isotope labeling of protein in the \textit{P. pastoris} expression system, we used Rho expressed in \textit{P. pastoris} as a model protein to optimize the culture conditions.\textsuperscript{26} The AATS isotope-labeled Rho expressed in \textit{P. pastoris} with different culture conditions was purified to homogeneity using Ni\textsuperscript{2+}-chelating chromatography and C18 reversed-phase HPLC. On the basis of SDS-PAGE, the AATS isotope-labeled Rho proteins produced in \textit{P. pastoris} were homogenous (data not shown). The yields of the AATS isotope-labeled Rho proteins produced in \textit{P. pastoris} were 2.8-18.9 mg/l. A platelet aggregation assay was used to examine their function in inhibiting platelet aggregation.\textsuperscript{26} The AATS isotope-labeled Rho inhibited platelet aggregation with the \textit{K}_i values of 70-87 nM, which were consistent with our reported \textit{K}_i value of 78 nM, which suggests that they retain their function.\textsuperscript{26}

Optimization of Culture Conditions

Preparation of AATS isotope labeling of protein often suffers from isotope scrambling arising from metabolic conversion of amino acids and low protein yield.\textsuperscript{9,13-18} Because we lacked a method to produce AATS isotope-labeled protein expressed in \textit{P. pastoris}, we used the AATS isotope minimal medium for \textit{E. coli} without adding glucose as our testing induction medium,\textsuperscript{18} as described in Materials and Methods. The general strategy for protein expressed in \textit{P. pastoris} requires a two-day induction period.\textsuperscript{26-33} However, the protein produced in this culture condition resulted in a low yield and a serious cross-labeling problem. The incorporation rate and protein yield of AATS isotope labeling of protein expressed in other expression systems are influenced by the composition of the medium, cell densities, and
induction time.\textsuperscript{13-18} We therefore optimized the culture conditions, including the concentrations of ammonium chloride, unlabeled amino acid, and \([\alpha-\text{^{15}N}]-\text{amino acid},\)
as well as the induction time of the protein expression, to increase the incorporation rate and protein yield. We used \([\alpha-\text{^{15}N}]-\text{Leu}\) to optimize the culture conditions because it is inexpensive and has a non-scrambling character found in other expression systems.\textsuperscript{13-18} The incorporation and scrambling rates were examined using mass or NMR spectroscopy.

The results of different culture conditions are summarized in Table 1. A concentration-dependent study of ammonium chloride with the range of 0-0.5 g/L showed that 0.25 g/L ammonium chloride was optimal for the highest incorporation rate. The medium in the absence of ammonium chloride produced protein with a high yield but a low incorporation rate. In addition, the protein incorporation rate decreased with an increase in the concentration of ammonium chloride up to 0.5 g/L.

A concentration-dependent study of unlabeled amino acid showed that the concentration of unlabeled amino acid used in the \textit{E. coli} expression system was optimal for the \textit{P. pastoris} culture condition. The study on the effects of medium composition on the incorporation rate of \([\alpha-\text{^{15}N}]-\text{Leu}\) showed that the concentrations of isotope-labeled amino acid played the most important role in introducing the isotope into proteins. The incorporation of \([\alpha-\text{^{15}N}]-\text{Leu}\) into protein was optimized at the concentration of 50 mg/L. To obtain high yields of protein, it was necessary to allow 24-36 h for induction time for protein expression. The incorporation rate started to decline after 36 h of induction, and protein expressed after 48 h induction showed scrambling.

\textbf{Labeling Rho with} \([\alpha-\text{^{15}N}]-\text{Asp, -Cys, -Gly, -Leu, -Lys, -Met, and Selenomethionin}]

After we obtained the culture condition for $[\alpha^{-15}N]$-Leu labeling of Rho expressed in *P. pastoris*, we used it to produce selective $[\alpha^{-15}N]$-Asp-, -Cys-, -Gly-, -Lys-, -Leu-, and Met-labeled Rho protein. Mass spectrometric and NMR measurements were used to examine the incorporation and scrambling rates of the AATS isotope. The mass spectra of unlabeled, $[\alpha^{-15}N]$-Leu-labeled, -Cys-labeled, and selenomethionine-labeled Rho are shown in Figure 1A-D, respectively. The experimental molecular weights of $[\alpha^{-15}N]$-Leu-, $[\alpha^{-15}N]$-Cys-, and selenomethionine-labeled Rho were 8419.1 ± 1 Da, 8429 ± 1 Da, and 8462.7 ± 1 Da, and they deviate less than 1 Da when compared with calculated values. The incorporation rates of the $[\alpha^{-15}N]$-labeled amino acids into Rho were calculated using mass spectra. For example, the experimental molecular weights of unlabeled and $[\alpha^{-15}N]$-Cys-labeled Rho were 8417 Da and 8429 Da, respectively (Figure 1C). The incorporation rate of 50% was calculated using the ratio of relative abundances of 8429 Da and 8400-8500 Da mass peaks. We also used 1D $^{15}$N-edited and -filtered NMR spectra to assess the incorporation rate with the resolvable C33 peak (data not shown). The resulting incorporation rate was ~45-55% that was similar to the results of mass spectrum. The scrambling rate was calculated using the ratio of relative abundances of the largest scrambling signal and the average labeled signals. In contrast to the mass spectrum of Rho, that of $[\alpha^{-15}N]$-Leu-labeled Rho showed heterogeneous species that may be the scramble-labeled Rho (Figures 1A-B). This is consistent with the NMR spectrum of $[\alpha^{-15}N]$-Leu-labeled Rho, which exhibited many small resonances from other residues, with ~5-10% scrambling rate (Figure 2A and Table II). The mass spectra of $[\alpha^{-15}N]$-Cys- and selenomethionine-labeled Rho showed that the predominant species were the unlabelled and labeled Rho (Figures
HCQC spectra of selective \([\alpha-^{15}\text{N}]-\text{Lys}\) and -Met-labeled Rho also exhibited patterns of well-dispersed, sharp resonances without any scrambling resonance (Figures 2B-C). The labeling of \([\alpha-^{15}\text{N}]-\text{Cys}\) had \(-45-55\%\) incorporation rate with \(-5-10\%\) scrambling rate from the side chains of Q35 and Q60, and all 12 cysteine resonances were observed in the HSQC spectrum (Table II and Figure 2D). In addition, the labeling of selenomethionine residue had \(-50-60\%\) incorporation rate, which was consistent with the 50-62\% incorporation rate of \([\alpha-^{15}\text{N}]-\text{Met}\) residue (Figure 1D). In addition to unlabelled and labeled peaks shown in Figure 1D, the mass spectrum of selenoMet-labeled Rho contained an 8512.7 Da peak, which may correspond to an oxidized product of diselenomethionine with the calculated value, 8511.\(^{39}\) Superimposition of HSQC spectra of \([\alpha-^{15}\text{N}]-\text{Cys}\), -Lys, -Leu, and -Met-labeled Rho with uniformly \(^{15}\text{N}\)-labeled Rho showed that they matched well with resonances (Figure 2E). In contrast, the labeling of \([\alpha-^{15}\text{N}]-\text{Asp}\) and -Gly amino acids had less than a 10\% incorporation rate with the scrambling levels >80\% (Table II). This is consistent with their scrambling character of \([\alpha-^{15}\text{N}]-\text{Asp}\) and -Gly amino acids found from the proteins expressed in other expression systems.\(^{17-18}\)

**Labeling Den with \([\alpha-^{15}\text{N}]-\text{Cys}, -\text{Leu}, -\text{Lys}, \text{and} -\text{Met}\)**

To test whether the obtained culture condition can be applied in labeling other proteins, we also labeled recombinant Den, a four disulfide-bonded protein expressed in *P. pastoris* with the correct fold. NMR spectroscopy was used to examine the level of isotope scrambling on labeled proteins and found that the culture condition was successfully applied to label Den with \([\alpha-^{15}\text{N}]-\text{Leu}, -\text{Lys}, -\text{Met, and} -\text{Cys}\) (Figures 3A-D). Their HSQC spectra exhibited a pattern of well-dispersed, sharp resonances that matched well with resonances of uniformly \(^{15}\text{N}\)-labeled Den (Figure 3). The labeling of Den with \([\alpha-^{15}\text{N}]-\text{Lys}\) and -Met exhibited no scrambling signals that were
consistent with those of AATS-labeled Rho (Figures 2B-C and 3B-C). In contrast, the labeling of Den with [α-15N]-Leu and -Cys produced 4-5% scrambling rates that were similar to the results of the labeling of Rho (Figures 2A, 2D, 3A, and 3D).
DISCUSSION

In the present study, we developed, for the first time, a protocol to produce AATS isotope-labeled Rho expressed in a P. pastoris system by optimizing the culture conditions, including the concentrations of ammonium chloride, unlabeled amino acid, and [α-^{15}N]-amino acid, as well as induction time. These culture conditions allowed us to overcome scrambling and low-yield problems and to produce [α-^{15}N]-Cys-, -Leu-, -Lys-, and -Met-labeled Rho with a 50-80% incorporation rate and protein yields of 9.5-13.4 mg/L. The analysis of 2D ^{15}N-^{1}H HSQC spectra showed that [α-^{15}N]-Cys-, -Leu-, -Lys-, and -Met-labeled Rho proteins have strong signals for the labeled amino acids with a 0-5% scrambling level. We also successfully applied the condition to label Den, which suggested that the described condition should be generally applicable to other proteins produced in the P. pastoris expression system. This culture condition should facilitate the AATS isotope labeling of proteins that can be used to simplify and confirm their NMR resonance assignment.

Protein labeling plays an important role in structural biology research, and protein structure determination using NMR and X-ray techniques usually requires labeling of the protein. The incorporation level of isotope labeling depends on the expression systems; therefore, the optimization of culture conditions and isotope sources to obtain high levels of isotope incorporation are essential. For example, there is substantial difference in incorporation levels of isotope labeling previously observed between P. pastoris vs. E. coli. Cells grown on an unlabeled carbon source a washed and re-suspended in carbon-labeled medium, resulted in >95% of carbon-labeled incorporation in E. coli. In contrast, P. pastoris grown on a glucose-containing medium and then switching to a methanol medium to initiate
induction via the AOX1 promoter, resulted in 70% (glucose) and 30% (methanol) isotope incorporation of carbon sources for induced protein.\textsuperscript{31}

To increase the incorporation level of AATS isotope labeling is still one of the most difficult tasks in protein expression.\textsuperscript{9,13-18} The principal difficulty associated with the preparation of AATS isotope-labeled proteins is the suppression of metabolic scrambling of the label into other amino acid types through the common metabolic pathways in the host cell.\textsuperscript{13} Although the culture condition for AATS isotope labeling of protein expressed in \textit{P. pastoris} for NMR study is not available, the use of yeast expression systems to incorporate selenomethionine for crystallographic studies has been reported.\textsuperscript{40-42} The levels of selenomethionine incorporation were 40-65\%, similar to our results of 60\% incorporation of the labeling of selenomethionine to Rho.\textsuperscript{40-42} Therefore, the culture condition should also facilitate producing selenomethionine-labeled proteins which can be used to perform multi-wavelength anomalous diffraction (MAD) experiments for X-ray protein analysis of proteins expressed in \textit{P. pastoris}.

In this study we overcame the scrambling problem and develop a protocol to produce AATS isotope labeling of protein in a \textit{P. pastoris} expression system. AATS isotope-labeled proteins are usually expressed in an auxotrophic strain, which cannot synthesize the amino acid to be labeled.\textsuperscript{17} However, auxotrophic strains usually have low levels of protein expression, and the yield of labeled proteins is often insufficient for structural analysis. In contrast, prototrophic strains are used for selective labeling with high protein yield and without constructing auxotrophic strains.\textsuperscript{43} Therefore, we used a prototrophic strain to optimize the culture condition of AATS isotope-labeled Rho in \textit{P. pastoris}. The possibility of producing AATS isotope-labeled proteins in the \textit{P. pastoris} prototrophic strain will attract more attention to its use as an expression
host for NMR and X-ray structure determination.

In conclusion, this is the first report to delineate the culture conditions for successful AATS isotope-labeling of proteins expressed in *P. pastoris* for NMR study. The methodology described herein for Rho has been applied to Den, suggesting that it should be generally applicable to other proteins produced in the *P. pastoris* expression system. Using the simple medium and general host/vector *P. pastoris* system, it should now be possible to produce a sufficient amount of AATS isotope-labeled recombinant proteins in *P. pastoris* for detailed biophysical characterization. The described culture conditions for proteins expressed in *P. pastoris* will facilitate protein structure determination using X-ray crystallography with selenomethionine-labeled proteins and using NMR spectroscopy with AATS isotope-labeled proteins.
ACKNOWLEDGMENT

We are grateful to Dr. Szecheng J. Lo for helpful discussion.
REFERENCES


for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for

11. Gardner KH, Kay LE. The use of $^2$H, $^{13}$C, $^{15}$N multidimensional NMR to study
the structure and dynamics of proteins. Annu Rev Biophys Biomol Struct

101-152.

13. Muchmore DC, McIntosh LP, Russell CB, Anderson DE, Dahlquist FW.
Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15

14. Archer SJ, Bax A, Roberts AB, Sporn MB, Ogawa Y, Piez KA, Weatherbee JA,
factor beta 1: NMR signal assignments of the recombinant protein expressed and
isotopically enriched using Chinese hamster ovary cells. Biochemistry
1993;32:1152-1163.

15. Lustbader JW, Birken S, Pollak S, Pound A, Chait BT, Mirza UA, Ramnarain S,
Canfield RE, Brown JM. Expression of human chorionic gonadotropin uniformly
labeled with NMR isotopes in Chinese hamster ovary cells: an advance toward

Jahnke W. Amino-acid-type selective isotope labeling of proteins expressed in
Baculovirus-infected insect cells useful for NMR studies. J Biomol NMR

17. McIntosh LP, Wand AJ, Lowry DF, Redfield AG, Dahlquist FW. Assignment of


35. Sutcliffe MJ, Jaseja M, Hyde EF, Lu X, Williams JA. Three-dimensional structure


42. Larsson AM, Stahlberg J, Jones TA. Preparation and crystallization of selenomethionyl dextranase from Penicillium minioluteum expressed in Pichia

Table I. Effects of the concentrations of ammonium chloride, non-labeled amino acids, and [α-^{15}N]-Leu, as well as induction time, on the yield and incorporation rate of selective [α-^{15}N]-Leu-labeled Rho

<table>
<thead>
<tr>
<th>NH₄Cl (g/L)</th>
<th>Non-labeled amino acids (x³)</th>
<th>[α-^{15}N]-Leu (mg/L)</th>
<th>Induction time (hours)</th>
<th>Yield (mg/L)</th>
<th>Incorporation rate (%)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>10</td>
<td>24</td>
<td>18.4</td>
<td>30</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>10</td>
<td>24</td>
<td>13.4</td>
<td>42</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>10</td>
<td>24</td>
<td>13.3</td>
<td>35</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
<td>10</td>
<td>24</td>
<td>18.9</td>
<td>11</td>
</tr>
<tr>
<td>0.25</td>
<td>0.5</td>
<td>10</td>
<td>24</td>
<td>16.1</td>
<td>16</td>
</tr>
<tr>
<td>0.25</td>
<td>0.75</td>
<td>10</td>
<td>24</td>
<td>14.7</td>
<td>29</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>24</td>
<td>13.4</td>
<td>42</td>
</tr>
<tr>
<td>0.25</td>
<td>1.25</td>
<td>10</td>
<td>24</td>
<td>12.2</td>
<td>39</td>
</tr>
<tr>
<td>0.25</td>
<td>1.5</td>
<td>10</td>
<td>24</td>
<td>12.6</td>
<td>38</td>
</tr>
<tr>
<td>0.25</td>
<td>2</td>
<td>10</td>
<td>24</td>
<td>12.2</td>
<td>32</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>5</td>
<td>24</td>
<td>12.1</td>
<td>19</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>24</td>
<td>13.4</td>
<td>42</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>20</td>
<td>24</td>
<td>17.6</td>
<td>50</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>50</td>
<td>24</td>
<td>13.4</td>
<td>80</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>100</td>
<td>24</td>
<td>10.0</td>
<td>73</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>18</td>
<td>2.8</td>
<td>41</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>24</td>
<td>13.4</td>
<td>42</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>30</td>
<td>16.6</td>
<td>41</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>36</td>
<td>17.3</td>
<td>39</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>42</td>
<td>15.2</td>
<td>36</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>10</td>
<td>48</td>
<td>6.3</td>
<td>29</td>
</tr>
</tbody>
</table>
\(^a\)X represents the concentrations of non-labeled amino acids described in Methods.
\(^b\)Incorporation rate is determined using mass measurement described in Methods.
Table II. Comparison of the numbers of amino acids and resonances obtained in HSQC spectra, yield, scrambling rate, and incorporation rate of \( [\alpha^{15}N] \)-amino acid-labeled Rho

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of Amino Acids</th>
<th>Number of Resonances</th>
<th>Yield (mg/L)</th>
<th>Scrambling Rate (%)</th>
<th>Incorporation Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( [\alpha^{15}N] )-Cys</td>
<td>12</td>
<td>12</td>
<td>9.5</td>
<td>4-10</td>
<td>45-55</td>
</tr>
<tr>
<td>( [\alpha^{15}N] )-Leu</td>
<td>2</td>
<td>2</td>
<td>13.4</td>
<td>5-10</td>
<td>70-80</td>
</tr>
<tr>
<td>( [\alpha^{15}N] )-Met</td>
<td>1</td>
<td>1</td>
<td>8.7</td>
<td>NO(^b)</td>
<td>50-62</td>
</tr>
<tr>
<td>Seleno-Met</td>
<td>1</td>
<td>-</td>
<td>9.7</td>
<td>ND(^c)</td>
<td>50-60</td>
</tr>
<tr>
<td>( [\alpha^{15}N] )-Lys</td>
<td>4</td>
<td>4</td>
<td>11</td>
<td>NO(^b)</td>
<td>45-55</td>
</tr>
<tr>
<td>( [\alpha^{15}N] )-Asp</td>
<td>6</td>
<td>15(^a)</td>
<td>12</td>
<td>&gt;100</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>( [\alpha^{15}N] )-Gly</td>
<td>7</td>
<td>18(^a)</td>
<td>14</td>
<td>&gt;80</td>
<td>ND(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Extra resonances are due to scrambling.

\(^b\)NO, not observed.

\(^c\)ND, not determined.
FIGURE LEGENDS

Figure 1. Mass spectra of (A) unlabeled, (B) [α-15N]-Leu-labeled, (C) [α-15N]-Cys-labeled, and (D) selenomethionine-labeled Rho. The experimental molecular weights of unlabeled, [α-15N]-Leu-labeled, [α-15N]-Cys-labeled, and selenomethionine-labeled Rho were 8417 Da, 8419.1 Da, 8429 Da, and 8462.7 Da, respectively.

Figure 2. 2D 15N-1H HSQC spectra of (A) [α-15N]-Leu-, (B) [α-15N]-Lys-, (C) [α-15N]-Met-, (D) [α-15N]-Cys-, and (E) 15N-labeled Rho. The protein concentrations were 0.5-1 mM. Correlation peaks are labeled according to residues type and sequence number. The resonances of the side chains were labeled with ‘s’ sign, and the peaks connected by lines correspond to Gln and Asn side chain NH2 group. The scrambling resonances were boxed. In Fig 2E, [α-15N]-Leu, [α-15N]-Lys, [α-15N]-Met, and [α-15N]-Cys were shown in green, blue, purple, and red, respectively.

Figure 3. 2D 15N-1H HSQC spectra of (A) [α-15N]-Leu-, (B) [α-15N]-Lys-, (C) [α-15N]-Met-, (D) [α-15N]-Cys-, and (E) 15N-labeled dendroaspin. The protein concentrations were 0.5-1 mM. Correlation peaks are labeled according to residues type and sequence number. The resonances of the side chains were labeled with ‘s’ sign, and the peaks connected by lines correspond to Gln and Asn side chain NH2 group. The vector-derived residues are labeled with asterisks, and the scrambling resonances were boxed.
Figure 2E
Figure 3B
Figure 3D