การตรวจการแสดงออกของยีน *AFP, albumin, TGF-β1* และ *Snail* จากเลือดของผู้ป่วยมะเร็งตับชนิด hepatocellular carcinoma ด้วยวิธี real-time RT-PCR เพื่อใช้เป็น molecular biomarkers: การศึกษาเบื้องต้น

AFP, Albumin, TGF-β1 and *Snail* Expression as Molecular Biomarkers in Peripheral Blood of Hepatocellular Carcinoma Patients by Real-Time RT-PCR: A Preliminary Study

ธีรยุทธ ปิ่นแก้ว[1], ยงยุทธ ศิริวัฒนอักษร[2], ชัชวาลย์ ศรีสวัสดิ์[1], สืบวงศ์ จุฑาภิสิทธิ์[2], ชยภัทร รัชตวิภาสนันท์[1], สุภาพร เวทีกุล[3] และ วรพรรณ ศิริวัฒนอักษร[1]*

Theerayuth Pinkaew[1], Yongyut Sirivatanauksorn[2], Chatchawan Srisawat[1], Suebwong Chuthapisith[2], Chayapat Ratchatawipasanan[1], Supaporn Waeteekul[3] and Vorapan Sirivatanauksorn[1]*

[1]ภาควิชาชีวเคมี; [2]ภาควิชาศัลยศาสตร์; [3]ภาควิชาสูติศาสตร์-นรีเวชวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพฯ 10700

[1]Department of Biochemistry; [2]Department of Surgery; [3]Department of Obstetrics and Gynecology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700

**Corresponding author: sivsr@mahidol.ac.th*

บทคัดย่อ

ผู้วิจัยได้ศึกษาการแสดงออกของยีน 4 ชนิด คือ *alpha-fetoprotein (AFP), albumin (ALB), transforming growth facter-beta1 (TGF-β1)* และ *Snail* เพื่อใช้เป็น molecular multimarker สำหรับการตรวจการกลับเป็นซ้ำของมะเร็งตับชนิด hepatocellular carcinoma ภายหลังการรักษาด้วยการผ่าตัด โดยการเจาะเลือดจากผู้ป่วย 28 คน และอาสาสมัครสุขภาพดี 8 คน นำมาสกัด RNA และวัดการแสดงออกของยีนด้วยเทคนิค real-time RT-PCR ผลการทดลองปรากฏว่า ผู้ป่วย 4 คน มีการแสดงออกของยีน *TGF-β1* สูงขึ้นหลังการผ่าตัด เมื่อติดตามต่อไปพบว่า 3 ใน 4 คนมีการกลับเป็นซ้ำของโรค จึงมีแนวโน้มที่เป็นไปได้ในการใช้ *TGF-β1* นี้ สำหรับตรวจการกลับเป็นซ้ำของมะเร็งตับในผู้ป่วยหลังได้รับการผ่าตัด

ABSTRACT

We studied the expression of four genes, *alpha-fetoprotein (AFP), albumin (ALB), transforming growth facter-beta1 (TGF-β1)* and *Snail*, as molecular multimarker in peripheral blood of hepatocellular carcinoma patients. Peripheral blood was collected from 28 HCC patients who underwent hepatic resection and 8 healthy normal volunteers, RNA was extracted and the expression was measured by real-time RT-PCR. Interestingly, the result showed that four patients have an increasing level of *TGF-β1* after surgery and almost all of them (three patients) got recurrence. Hence, *TGF-β1* might be a promising marker for the detection of recurrent HCC.

คำสำคัญ: ยีน *AFP*, ยีน *ALB*, ยีน *TGF-β1*, ยีน *Snail*, เฮปปาโตเซลลูลาร์ คาร์ซิโนมา, เรียล-ไทม์ อาร์ที-พีซีอาร์

Keywords: *AFP, ALB, TGF-β1, Snail*, hepatocellular carcinoma, real-time RT-PCR

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common life-threatening affected malignancies. It is the second cause of cancer death in males and the sixth in females (Jemal A, 2011). Generally, cirrhosis, chronic repetitive hepatic injury, chronic alcohol consumption, chronic infection with the hepatitis virus B and C and exposure to aflatoxin B1 are associated with the prevalence of HCC and represent major underlying factors predisposing the development of HCC (Mckillop, 2006). The treatment of HCC at present are still limited to patients who have less advanced disease. Hepatic resection is the treatment of choice with a 5-year survival rate of 50% and a 70% recurrence rate (Sherman M, 2008). Also, we still have lack of knowledge about its molecular, cellular and environmental mechanism which determines the pathogenesis of HCC. Finding sensitive markers for early diagnosis and monitoring of post-operative recurrence is the most important thing for sufficient treatment. Many studies confirm that detecting a single marker in peripheral blood is less effective and multimarker RT-PCR is recommended to improve sensitivity and specificity of the assay (Yao DF, 2007, Paterlini-Brechot P, 2007, Chiappini F, 2012).

In this study, we aim to detect multiple combination markers in peripheral blood by using real-time RT-PCR. *Alpha-fetoprotein (AFP), albumin (ALB), transforming growth factor-beta1 (TGF-β1)* and *Snail* are employed as markers in our study. We expect that the result from this study would facilitate in establishing an appropriate combination markers for early detection of recurrent HCC after hepatic resection.

MATERIALS AND METHODS

RNA was extracted from 28 early stage HCC patients and 8 healthy normal volunteers. The quantity of extracted RNA was acheived by measuring the absorbance at 260 nm (A_{260}) using the Nanophotometer™. For the quality of extracted RNA, we assessed by Bioanalyzer™ which reports RNA's quality in term of RNA intregrity number (RIN). Then cDNA was synthesized using the Quantitect Reverse Transcription Kit. We evaluated the expression of *AFP, ALB, TGF-β1* and *Snail* mRNA by real-time reverse transcription polymerase chain reaction (real-time RT-PCR). *GAPDH* was used as the normalising gene and HepG2, a HCC cell lines, was used as the positive control in our experiment.

RESULTS AND DISCUSSION

The threshold cycles (Ct) which are results from real-time RT-PCR of each cDNA will be calculated to mean normalised expression (MNE) value before use in statistical analysis. The formula was shown below.

$$MNE = \frac{2^{(Ct\ ref.\ mean)}}{2^{(Ct\ target\ mean)}}$$

In all samples, no threshold cycles were measured from cDNA of *AFP*, so we could not calculate MNE value of this gene. However, in HepG2 cell lines, we got the reliable threshold cycles of *AFP* in every experiment. The result from the melting temperature of PCR product (Tm) also confirmed that the threshold cycles of *AFP* from HepG2 cell lines were the same product

because they had melted at the same temperature. The study of *AFP* mRNA as a marker of circulating tumour cells is controversial. There were some studies proposed that they could quantify *AFP* mRNA in peripheral blood (PB) of HCC patients and *AFP* mRNA was a valuable marker. On the other hand, numerous studies claimed that the false positive results could be obtained using *AFP* mRNA. Additionally, many studies concluded that the measurement of *AFP* mRNA in PB of patients with HCC was not a clinically relevant method for determining recurrent and prognosis of HCC and might not be used as a good molecular marker for recurrent HCC (Chiappini F, 2012).

For *ALB* gene, we found that *ALB* expression in PB of patients was significantly increased in immediately post-operative samples compared with pre-operative (*p-value = 0.009*). Among 28 patients, 21 patients had an increase in *ALB* expression after an operation, 4 patients had the level down and 3 patients had no change occur. Nevertheless, it was provocative about using *ALB* mRNA as a molecular marker for HCC. Many studies confirmed that hepatocytes can be released into the circulation by surgical manipulation and the *ALB* mRNA might be from those hepatocytes (Wong IH, 1999). Interestingly, we also found that patients with high expression of *ALB* before surgery had recurrence within three to six months after operation (75%).

The level of *TGF-β1* mRNA of 24 patients was decreased in post-operative samples compared with pre-operative ones (*p = 0.003*). Only 4 patients had the level increased after surgery. Interestingly, among these 4 patients, 3 patients became recurrence (75%). *TGF-β1* is multifunctional polypeptides that can influence tumour cell by directly binding to it receptor or by influencing the peritumouring milieu. Although, *TGF-β1* is considered to be a major negative regulatory factor in the liver cell proliferation, it may exert paradoxical effects on the tumour cells by a variety of mechanisms (Abou-Shady M, 1999, Min AL, 2009). The expression level increases after surgery might be from circulating tumour cells (CTCs) which will cause recurrence in the future.

For the *Snail* gene, our results showed no significant difference between patients and normal healthy volunteers. The expression level of *Snail* mRNA also revealed no difference between groups of patients at different follow-up time. It was one of the key factors in epithelial-mesenchymal transition (EMT) process which cause CTCs spread out from primary tumour and cause metastasis (Min AL, 2009). We selected this gene because *Snail* was significantly expressed in several proteomic analyses of HCC tissue. However, it might be because of a small number of samples which made our results showed no significant difference.

CONCLUSION

We conclude that only *TGF-β1* may be a promising marker for the detection of recurrence hepatocellular carcinoma after surgery. Seventy-five percent of patients who have increase *TGF-β1* expression after operation have had recurrence disease. However, the study of other molecular markers for HCC and the role of multimarker are still imperative.

ACKNOWLEDGEMENTS

This study was supported by the Siriraj Hospital Development Scholarship and Siriraj Grant for Research Development and Medical Education, Faculty of Medicine, Siriraj Hospital, Mahidol University.

REFERENCES

Abou-Shady M, Baer HU, Friess H, Berberat P, Zimmermann A, Graber H, et al. Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. American journal of surgery. 1999;177(3):209-15.

Chiappini F. Circulating tumor cells measurements in hepatocellular carcinoma. International journal of hepatology. 2012;2012:684802.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61(2):69-90.

McKillop IH, Moran DM, Jin X, Koniaris LG. Molecular pathogenesis of hepatocellular carcinoma. The Journal of surgical research. 2006;136(1):125-35.

Min AL, Choi JY, Woo HY, Kim JD, Kwon JH, Bae SH, et al. High expression of Snail mRNA in blood from hepatocellular carcinoma patients with extra-hepatic metastasis. Clinical & experimental metastasis. 2009;26(7):759-67.

Paterlini-Brechot P, Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. Cancer letters. 2007;253(2):180-204.

Sherman M. Recurrence of hepatocellular carcinoma. The New England journal of medicine. 2008;359(19):2045-7.

Wong IH, Lau WY, Leung T, Yeo W, Johnson PJ. Hematogenous dissemination of hepatocytes and tumor cells after surgical resection of hepatocellular carcinoma: a quantitative analysis. Clinical cancer research: an official journal of the American Association for Cancer Research. 1999;5(12):4021-7.

Yao DF, Dong ZZ, Yao M. Specific molecular markers in hepatocellular carcinoma. Hepatobiliary & pancreatic diseases international : HBPD INT. 2007;6(3):241-7.