High Expression of EGFRvIII Predicts Poor Prognosis in Cholangiocarcinoma Patients

การแสดงออกที่สูงขึ้นของ EGFRvIII บ่งชี้ถึงการพยากรณ์โรคที่ไม่ดีในผู้ป่วยมะเร็งท่อน้ำดี

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ABSTRACT

Cholangiocarcinoma (CCA) is a malignant tumor originating in bile duct epithelial cells. The high incidence of CCA worldwide is reported in northeast Thailand where it is a major health problem. EGFRvIII is the most common EGFR mutation and is usually associated with a poor prognosis for cancer patients. We therefore hypothesize that EGFRvIII may be involved in poor outcome of CCA patients. In the present study, we explored the expression of EGFRvIII in 94 cases of Ov-associated CCA using tissue sections. EGFRvIII had a low expression in 35% of the samples and a high expression in while 65% . These expression data were correlated with clinico-pathological data. We found high expression of EGFRvIII was significantly associated with poor prognosis of patients. Therefore, the mutated form of EGFR (EGFRvIII) might be serve as a drug target in order to improve CCA treatment.

บทคัดย่อ

โรคมะเร็งท่อน้ำดีคือมะเร็งที่เกิดจากเซลล์เยื่อบุทางเดินน้ำดี ซึ่งพบว่ามีอุบัติการณ์สูงและเป็นปัญหาทางด้านสาธารณสุขในภาคตะวันออกเฉียงเหนือของประเทศไทย EGFRvIII เป็นการกลายพันธุ์ที่พบได้บ่อยของ EGFR และมักมีความเกี่ยวข้องกับการพยากรณ์โรคที่ไม่ดีของผู้ป่วย ดังนั้นผู้วิจัยจึงตั้งสมมติฐานว่า การแสดงออกของ EGFRvIII อาจมีความเกี่ยวข้องกับการพยากรณ์โรคที่ไม่ดีในผู้ป่วยมะเร็งท่อน้ำดีด้วย ในการศึกษานี้ผู้วิจัยได้ทำการศึกษาการแสดงออกของ EGFRvIII ในชิ้นเนื้อผู้ป่วยโรคมะเร็งท่อน้ำดี จำนวน 94 ราย พบว่าผู้ป่วยมีการแสดงออกของ EGFRvIII ในระดับต่ำ 35 เปอร์เซ็นต์ ขณะที่ 65 เปอร์เซ็นต์มีการแสดงออกในระดับสูง เมื่อนำระดับการแสดงออกของ EGFRvIII มาวิเคราะห์ร่วมกับข้อมูลทางคลินิกของผู้ป่วย พบว่าการแสดงออกสูงของ EGFRvIII มีความสัมพันธ์กับการพยากรณ์โรคที่ไม่ดีในผู้ป่วย ดังนั้น EGFRvIII อาจจะสามารถนำมาใช้เป็นเป้าหมายของยาเพื่อที่จะปรับปรุงการรักษาโรคมะเร็งท่อน้ำดีต่อไป

Keywords: EGFR, EGFRvIII, Ov-associated Cholangiocarcinoma
คำสำคัญ: อีจีเอฟอาร์ การกลายพันธุ์ของอีจีเอฟอาร์ มะเร็งท่อน้ำดีที่เกี่ยวข้องกับการติดเชื้อพยาธิใบไม้ในตับ

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Introduction

Cholangiocarcinoma (CCA) is a cancer that originates from bile duct epithelial cells. Although, many factors that can cause CCA have been reported worldwide, *Opisthorchis viverrini* (Ov) infection is the a major risk factor for developing CCA in northeast Thailand (Sripa and Pairojkul, 2008). Our group has recently reported alterations in gene expression in CCA. Among these, EGFR was prominently activated in both CCA tissues and cell lines (Dokduang et al., 2013). This result leads us to focus on the alteration of EGFR in CCA as EGFR is involved in many cellular processes. Moreover, the alteration of EGFR is commonly involved in cancer progression. EGFRvIII (de2-7EGFR or Δ801EGFR) is the most common of EGFR deletion in the extracellular domain of EGFR, which is constitutively phosphorylated (Pedersen et al., 2001). This truncated receptor is a tumor-specific mutation that is caused by an in-frame deletion of exons 2-7 in mRNA that removes 801 base pairs or 267 amino acids (Gan et al., 2013). This mutation has been reported to drive cancer progression and is usually associated with a poor prognosis for the patients (Gan et al., 2013). There are many reports demonstrating the role of EGFRvIII in contributing to cancer progression (Nagane et al., 1996; Narita et al., 2002; Wheeler et al., 2010; Yamoutpour et al., 2008). Although, EGFRvIII has been studied in several cancers, its the role in Ov-associated CCA is still unknown. In the present study, we therefore aimed to investigate the association of EGFRvIII with the clinico-pathological outcomes of CCA patients.

Objective of the study

This study aimed to investigate the expression of EGFRvIII in Ov-associated cholangiocarcinoma (CCA) and determine its association with the patient's prognosis.

Material and methods

Human CCA tissues

The paraffin embedded Ov-associated CCA tissues were obtained from the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. The study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University (HE571283). Informed consent was obtained from individual patients.

Immunohistochemical staining

Immunohistochemical staining was performed using archived human liver tissues as detailed above. We followed the standard protocol of immunoperoxidase staining for detecting EGFRvIII expression in Ov-associated CCA liver tissues. Briefly, the sections of Ov-associated CCA liver tissues were de-paraffinized and rehydrated stepwise using xylene follow by 100%, 90%, 80% and 70% ethanol. Antigen retrieval was performed by microwave cooking with 10 mM citrate buffer pH 6 and 0.05% Tween20 for 10 minutes. The sections were treated with 3% hydrogen peroxide and 10% skim milk to block endogenous hydrogen peroxide activity and nonspecific binding for

30 minutes at each step. The tissue sections were incubated with primary antibody (anti-EGFRvIII antibody; dilution 1:100) for 1 hour at room temperature followed by 4°C overnight. Next, sections were washed in phosphate buffered saline (PBS) with 0.1% tween20 and incubated with secondary antibody (DakoEnVision) for 1 hour. After that, the signal was developed with a 3, 3'diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Inc., Burlingame, CA) for 5 minutes, then counterstained with Mayer's haematoxylin. Finally, the sections were dehydrated stepwise using 70%, 80%, 90%, 100% ethanol and xylene and mounted with permount. After that, the stained sections were magnification under microscope.

Immunohistochemical grading

Grading criteria are based on the intensity and frequency of staining (Namwat et al., 2011). The intensity of EGFRvIII expression was divided into four groups: 0 = negative; +1 = weak expression; +2 = moderate expression; +3 = strong expression. The frequency of EGFRvIII expression was divided into four groups: 0 = negative; +1 = 1–25 %; +2 = 26–50 %; +3 > 50 %. Staining score was calculated by multiplying intensities and frequencies in each case. Low and high of EGFRvIII expression depended on grading scores and were divided into two groups: EGFRvIII expression level scores $<$ 6 = low and EGFRvIII expression level scores $\geq$ 6 = high.

Statistical analysis

The association between EGFRvIII expression with clinico-pathological data of CCA patients was analyzed by chi-square test (Statistical Package for the Social Science; SPSS software v.16). Survival analysis was performed using Kaplan-Meier (SPSS software v.16). The result was considered to be significant at P-value < 0.05.

Results

Patient characteristics

A total of 94 Ov-associated CCA tissue sections were studied, 62% from males and 38% from females. The age of the patients ranged from 33 to 76 years (median = 55 years). In this study, histological types were classified as papillary CCA (38%) and non-papillary CCA (62%). All patients had advanced stage cancer and metastasis was observed in 49%. In addition, the median of survival time after surgery was divided into $<$ 452; 47 cases (50%) and $\geq$ 452; 47 cases (50%) (Table. 1).

Immunohistochemical staining

The expression of EGFRvIII was explored in Ov-associated CCA tissues using immunohistochemical staining. Low and high expression of EGFRvIII were demonstrated in figure 1A. From the immunohistochemical analysis, low EGFRvIII was found in 35% of samples while 65% showed high expression (Table. 1). High expression of EGFRvIII was significantly associated with non-papillary type (P = 0.002), metastasis status (P = 0.032) and

survival time after surgery of CCA patients (P = 0.030) (Table. 1). Moreover, survival analysis showed that high expression of EGFRvIII was significantly correlated with shorter survival of CCA patients (P = 0.049) (figure. 1B).

Table 1 The association between EGFRvIII expression with pathological features of Ov-associated CCA patients analyzed by chi-square test (SPSS software v.16). Statistical significance was considered to be present at $^{*}P$-value < 0.05, $^{**}P$-value < 0.01.

Variable		No. of patients (%)	EGFRvIII expression		
			Low	High	*P*-value
Gender	Male	58(62%)	19	39	0.657
	Female	36(38%)	14	22	
Age	<55	45(48%)	19	26	0.198
	≥55	49 (52%)	14	35	
Histological type	Papillary	36(38%)	20	16	0.002**
	Non-papillary	58(62%)	13	45	
Metastasis status	Non-metastasis	48(51%)	22	26	0.032*
	Metastasis	46(49%)	11	35	
Survival time (day)	<452	47(50%)	11	36	0.030*
	≥452	47(50%)	22	25	
Percentage (%)			35	65	

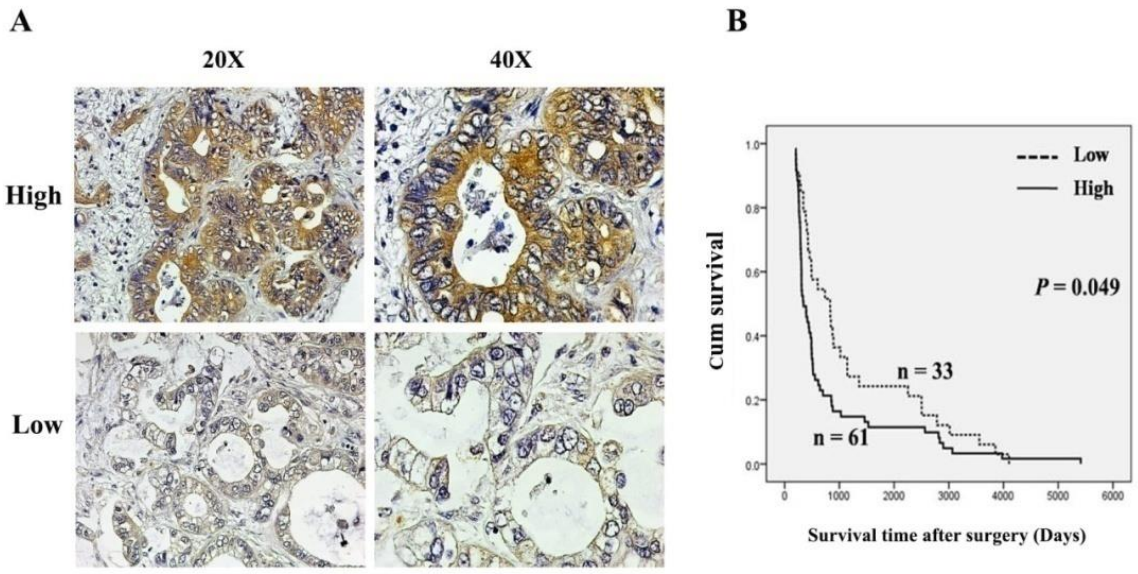

Figure 1 Expression of EGFRvIII in Ov-associated CCA tissues and survival analysis. Ov-associated CCA tissues were stained with anti-EGFRvIII antibody (dilution 1:100). A, shows low and high expression of EGFRvIII. B, shows the survival analysis of EGFRvIII.

Discussion and conclusion

The alteration of EGFR has been widely studied in many cancer types, including CCA (Gan et al., 2013; Harari, 2004). EGFRvIII is the most common EGFR mutation and the expression of EGFRvIII varies between different cancer types (Gan et al., 2013). Most of the reports demonstrated that EGFRvIII expression is associated with a poor prognostic for patients (Gan et al., 2013). Thus, we hypothesized that the expression of EGFRvIII in CCA may indicate a poor outcome in these patients. In the present study immunohistochemical staining was used to examine the expression of EGFRvIII in Ov-associated CCA tissues. The results showed that the expression of EGFRvIII was high in these tissues. Moreover, high expression of EGFRvIII was significantly associated with non-papillary type, metastasis status and in shorter survival of CCA patients. Our finding is consistent with previous study that showed high expression of EGFRvIII was associated with poor prognosis of oral cancer patients (Chang et al., 2013). Moreover, the role of EGFRvIII to contribute cancer progression has been demonstrated in both *in vitro* and *in vivo* studies (Sok et al., 2006). Our data suggest that EGFRvIII might play an important role in CCA progression. Therefore, EGFRvIII may be of value as a drug target for improve CCA therapy.

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