14-3-3 Zeta Protein Expression Profile in the Opisthorchiasis-Associated Cholangiocarcinoma

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ABSTRACT

14-3-3 zeta is one of the 14-3-3 family of proteins expressed in eukaryotic cells. They play important roles in various cellular functions. Overexpression of 14-3-3 zeta is involved in tumor development and progression and is found in many cancers. We investigated the expression pattern of 14-3-3 zeta using immunohistochemistry in cholangiocarcinoma (CCA) that was correlated with liver fluke (*Opisthorchis viverrini; Ov*) infection which is the major public health problem in Northeast of Thailand. Our results showed that 14-3-3 zeta expression was increased in a Hamster group with CCA, induced by Ov infection in combination with N-nitrosodimethylamine (NDMA), compared to the untreated groups. This was demonstrated by higher immunohistochemical scores in biliary cells as carcinogenesis progressed. We suggest that 14-3-3 zeta might be an oncogenic factor that regulates the genesis of Ov-associated CCA.

Keywords: 14-3-3 zeta, *Opisthrochiasis*, Cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is a type of cancer which produces the highest mortality rate of any cancer reported in Northeast Thailand. Etiology of CCA in Thailand has correlated with liver fluke (*Opisthorchis viverrini*) infection that causes chronic inflammation (Sripa *et al.*, 2008; Songserm *et al.*, 2009) resulting in alteration of gene expression during cholangiocarcinogenesis (Pinlaor *et al.*, 2003; Loilome *et al.*, 2006).

The 14-3-3 family of proteins are highly conserved, dimeric proteins that are localized in nucleus and cytoplasm in eukaryotic cells. In mammals, there are seven isoforms encoded from seven different genes including beta (β), epsilon (ε), eta (η), theta (τ), gamma (γ), sigma (σ) and zeta (ζ). They function through serine/threonine phosphorylation binding motifs of their target proteins (Neal *et al.*, 2009) leading to regulating multiple cellular processes such as cell cycle progression, cell signaling, cytoskeletal dynamics, cell metabolism, autophagy and cell proliferation (Hermeking, 2003). One of the 14-3-3 family of proteins, 14-3-3 zeta has been determined as an oncogenic isoform and overexpression of 14-3-3 zeta is associated with multiple cancer types and regulates various pathways that promote cancer initiation and progression (Neal *et al.*, 2010). Overexpression of 14-3-3 zeta found in many cancers that was associated with short survival rate of patients such as lung cancer (Fan *et al.*, 2007), breast cancer (Neal *et al.*, 2009), and non Ov associated CCA (Zhang *et al.*, 2015).

In this study, we hypothesized that the 14-3-3 zeta may be involved in cholangiocarcinogenesis.

Objective of the study

This study investigated the expression profiles of 14-3-3 zeta protein in a hamster model of induced CCA caused by Ov metacercariae infection combined with *N*-nitrosodimethylamine (NDMA).

Methodology

Animal model

The protocol was approved by Animals Ethic Committee, Faculty of Medicine, Khon Kaen University (approval number AEKKU 23/2555). Syrian golden hamsters were infected with Ov metacercariae combined with *N*-nitrosodimethylamine (NDMA) in order to induce CCA (Loilome *et al.*, 2006). The hamsters, ranging from 6 to 8 weeks of age, were divided into 2 groups: Group 1 remained untreated; Group 2 was treated with 50 Ov metacercariae combined with NDMA (Sigma, St. Louis, MO, USA) (12.5 ppm given in drinking water) for 8 weeks. Each hamster received approximately 0.166–0.04 mg/day of NDMA. Four liver tissue sections were fixed in 10% buffered-formalin. The fixed tissues were embedded in paraffin and then serially dissected into 4 µm thick slides for immunohistochemical analysis.

Immunohistochemistry staining

The localization of 14-3-3 zeta on liver section of hamster CCA tissues was detected by Immunohistochemistry (IHC) using a rabbit anti-14-3-3 zeta antibody (Cambridge, England) with a dilution of 1:50 for 1 hour and then incubation with

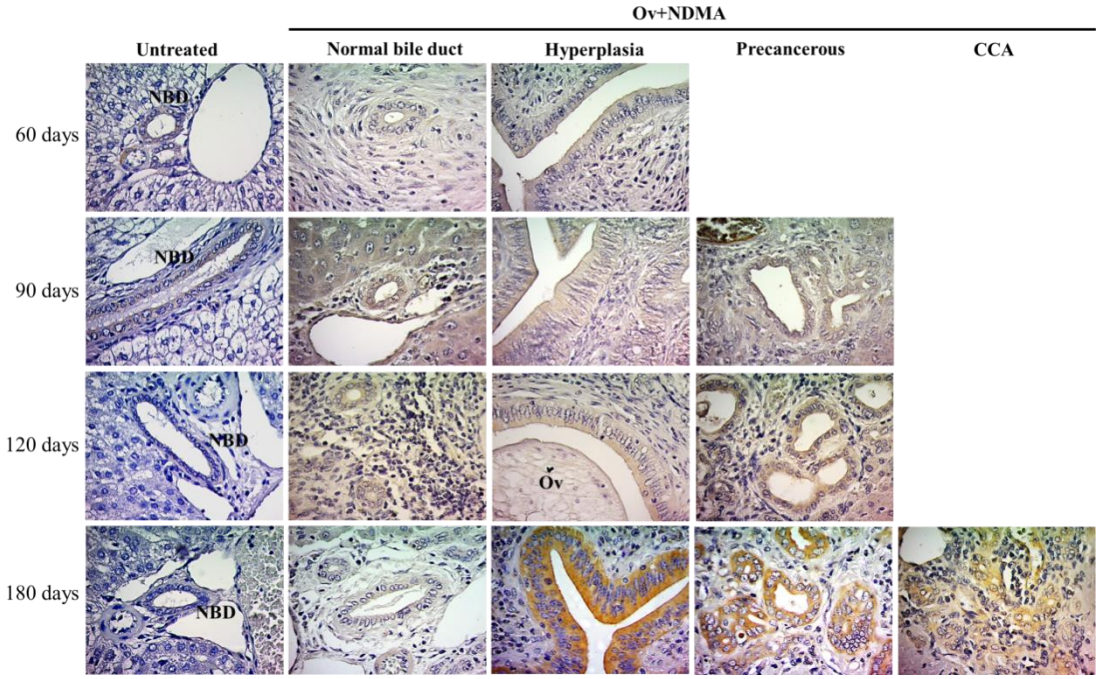

Figure 1 Representative immunohistochemical staining of 14-3-3 zeta expression in liver section treated with *Opisthorchis viverrini* (Ov) and *N*-nitrosodimethylamine (NDMA)-induced CCA hamster models for 60, 90, 120 and 180 days (x400). NBD; normal bile duct, OV; *O. viverrini*

peroxidase conjugated Envision secondary antibodies (DAKO). Peroxidase activity was then visualized with diaminobenzidine (DAB) solution and counterstained by hematoxylin. The stained sections were reviewed under a bright- field microscope.

Immunohistochemical grading

We performed immunohistochemical grading based on intensity and proportion. The staining intensity of 14-3-3 zeta was scored as follows: negative (0), low (+1), moderate (+2), or high (+3). The proportion was scored based on the percentages of stained cells as follows: negative (0), less than 25% (+1), 25-50% (+2), more than 50% (+3). The IHC score (0-9) was assigned by multiplying the intensity score by the proportion score. The scoring of 14-3-3 zeta expression was assigned as follows: less than the median of IHC score (+), equal to IHC score (++), and more than IHC score (+++).

Results

The expression of 14-3-3 zeta in induced CCA hamster tissues was determined by immunohistochemical staining. The scoring was performed by immunohistochemical grading based on intensity and frequency that represented in brown. We examined 14-3-3 zeta expression in hamster tissues of the untreated group and the treated group (Ov+NDMA) for 60, 90, 120 and 160 days post infection (p.i.) (figure 1). The results showed that 14-3-3 zeta was weakly expressed in tissues from the normal bile duct taken from the untreated group at 60 and 90 days p.i and no expression was found in tissues at 120 and 180 days p.i.. In the Ov+NDMA

treated group, 14-3-3 zeta was weakly expressed in the adjacent normal bile ducts. 14-3-3 zeta expression was slightly increased in hyperplasia and dysplastic of 120 days p.i.. 14-3-3 zeta was strongly expressed in hyperplasia, dysplastic lesion and cancer tissues at 180 days p.i. (figure 1). The intensity score of immunohistochemical staining of 14-3-3 zeta in hamster tissues is shown in table 1.

Table 1 The scoring of immunohistochemical staining of 14-3-3 zeta in hamster tissues

Days	Untreated	Treated			
	NBD	NBD	HP	PC	CCA
60	+	-	+		
90	+	+	++	++	
120	-	++	++	++	
180	-	-	+++	+++	+++

+, ++, +++; the scoring of 14-3-3 zeta expression were assigned as follows: less than the median of IHC score (+), equal to IHC score (++), and more than IHC score (+++).

-; no 14-3-3 zeta expression

Treated; Ov metacercariae infection combined with *N*-nitrosodimethylamine (NDMA), NBD; normal bile duct, HP; hyperplasia, PC; precancerous, CCA; cholangiocarcinoma

Discussion and Conclusions

Overexpression of 14-3-3 zeta is demonstrated in many cancers and plays important roles in the tumorigenesis and progression. This study demonstrated that the upregulation of 14-3-3 zeta expression was detected in hyperplasia, dysplasia and CCA tissues of Ov plus NDMA-induced CCA hamster indicating that 14-3-3 zeta expressed along the genesis of CCA. It has been proved that 14-3-3 zeta functions as an oncogenic factor that regulates tumor growth and metastasis (Zhang *et al.*, 2015). Increased 14-3-3 zeta in CCA genesis might be triggered by chronic inflammation (Kasinski *et al.*, 2014). Our finding suggests that 14-3-3 zeta is involved in Ov-associated cholangiocarcinogenesis and its overexpression is possibly a potential prognostic indicator of CCA.

Acknowledgements

We would like to acknowledge Robert Smith for editing this proceeding. This study was supported by the Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University.

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