ANALYSIS OF THE *REG1α* AND *REG1β* GENE TRANSCRIPTS IN PATIENTS WITH FIBROCALCULOUS PANCREATOPATHY

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Abstract. Fibrocalculous pancreatopathy is a form of diabetes, associated with tropical chronic calcific pancreatitis, in which islet β-cell loss and pancreatic stone formation are found. It is likely to be a multifactorial disease with both genetic and environmental components. Regenerating (*reg*) gene encodes protein that has been involved in pancreatic lithogenesis and the regeneration of islet cells and therefore the abnormality of *reg* genes could be associated with fibrocalculous pancreatopathy. In this study, *reg1α* and *reg1β* mRNAs were isolated from peripheral blood lymphocytes obtained from 16 patients with fibrocalculous pancreatopathy, 42 patients with type 1 diabetes, 37 patients with type 2 diabetes, and 22 normal controls. mRNAs were amplified by reverse-transcription polymerase chain reaction (RT-PCR) and analysed by a single strand conformation polymorphism (SSCP) technique. The *reg1α* and *reg1β* mRNAs were isolated, indicating the ectopic expression of these genes in peripheral blood lymphocytes; however, variation among mobility patterns was not observed in the SSCP analysis of the RT-PCR products. The results indicated that there was no abnormality of the *reg1α* and *reg1β* mRNAs obtained from the study groups.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It is a major cause of morbidity and mortality and is increasing in prevalence in many areas of the world (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). Fibrocalculous pancreatopathy, formerly *fibrocalculous pancreatic diabetes* (FCPD), is a unique form of diabetes secondary to the non-alcoholic, chronic calcific pancreatitis seen in tropical developing countries. (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997; Khan and Ali, 1997; Mohan *et al*, 1998). The disease is marked by its triad of cardinal findings: abdominal pain, pancreatic calculi, and diabetes.

The pathogenic mechanisms of FCPD remain unclear. Various factors have been suggested, including malnutrition, consumption of cyanide-containing foods (*eg* cassava), oxidant stress, antioxidant deficiency, and genetic factors. However, controversy still surrounds the role of these factors (Vannasaeng *et al*, 1986; Castano and Eisenbarth, 1990; Yajnik *et al*, 1990; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997; Mohan *et al*, 1998).

The pancreatic regenerating (*reg*) genes are expressed in the exocrine cells and regen-

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erating islet β-cells of the pancreas. It has been suggested that the activation of the type I *reg* gene is a stage in pancreatic β cell regeneration (Okamoto, 1999). Reg protein was also found to be present in the exocrine pancreas; it was associated with islet regeneration, an increase in the β-cell mass, and the amelioration of diabetes in animal models (Terazono *et al*, 1988; Bone *et al*, 1994; Watanabe *et al*, 1994; Baeza *et al*, 1996; Zenilman *et al*, 1996; Okamoto, 1999).

The human *reg* 1 genes, *reg1α* and *reg1β*, are located on chromosome 2p12. Each *reg* gene spans approximately 3.0 kb and is composed of six exons. *Reg1α* and *reg1β* have 70% homology, which differs in 22 amino acids (Terazono *et al*, 1988; Moriizumi *et al*, 1994; Watanabe *et al*, 1994; Zenilman *et al*, 1996). However, *reg1β* was expressed only in the pancreas while *reg1α* was also found to be expressed at a lower level in the gastric mucosa and the kidney. *Reg1α* gene encodes a 166 amino acid protein which was found to be identical to pancreatic-stone protein, or pancreatic-thread protein (lithostathine) (Watanabe *et al*, 1990), which might be involved in pancreatic lithogenesis.

It may be hypothesized that defects of *reg1α* and *reg1β* genes are associated with the pathogenesis of fibrocalculous pancreatopathy, which features both β-cell loss and pancreatic stone formation (Yajnik *et al*, 1992; 1997; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997; Khan and Ali, 1997; Mohan *et al*, 1998). We recently reported on a study of *reg1α* and *reg1β* genomic DNA in patients with fibrocalculous pancreatopathy: only polymorphisms of the *reg1α* gene and some mutations of the *reg1β* gene were found and none was associated with the disease (Banchuin *et al*, 2002). However, the abnormality of any gene can also be at the transcription step; it would be interesting to study *reg1α* and *reg1β* gene transcriptions in patients with such abnormalities. Since it is not possible to obtain islet β-cells from the pancreatic tissue of patients who do not require surgical treatment, we chose peripheral blood lymphocytes as a source of cells for the study of *reg1α* and *reg1β* gene transcriptions; illegitimate transcriptions of various tissue-specific genes, including *PKD1* and *factor VIII* genes, have been found in correctly-processed human peripheral blood lymphocytes (Chelly *et al*, 1989; Thongnoppakhun *et al*, 1999; Akkarapatumwong *et al*, 2000).

In this study, we examined the illegitimate transcriptions of *reg1α* and *reg1β* genes in peripheral blood lymphocytes and determined whether there was any abnormality of *reg1α* and *reg1β* gene transcriptions in patients with fibrocalculous pancreatopathy.

MATERIALS AND METHODS

Subjects

A total of 95 subjects (16 patients with fibrocalculous pancreatopathy, 42 patients with type 1 diabetes, and 37 patients with type 2 diabetes) were recruited from three hospitals in Bangkok and one hospital in Ubon Ratchathani, Thailand. Thirty-nine patients were admitted to or attended the diabetic clinics of Siriraj Hospital; 49 were patients of Rajavithi Hospital; 3 were patients of Phyathai 2 Hospital, Bangkok; 4 were patients of Sappasitthiprasong Hospital, Ubon Ratchathani.

Fibrocalculous pancreatopathy was diagnosed according to previously-established criteria, including: the onset of diabetes under 30 years of age and pancreatic calcification on plain abdominal X-ray; a requirement for insulin treatment since the onset of diabetes without ketonuria or ketosis despite the withdrawal of insulin for several weeks (Vannasaeng *et al*, 1986). These patients had no history of alcohol ingestion or gall bladder disease; they formed a group which comprised 7 males (aged from 28 to 54 years) and 9 females (aged from 34 to 51 years).

Type 1 diabetes was diagnosed on the basis of hyperglycemia that warranted treatment with insulin and a history of at least one episode of ketoacidosis on the withdrawal of

insulin or a deficient or absent C-peptide response following a glucagon stimulation test (Vannasaeng *et al*, 1986). This group of subjects comprised 13 males (aged from 14 to 44 years) and 29 females (aged from 12 to 52 years).

Patients who did not meet the criteria for fibrocalculous pancreatopathy and type 1 diabetes as described above were diagnosed as having type 2 diabetes: this group comprised 8 males (ages ranging from 30 to 79 years) and 29 females (ages ranging from 37 to 75 years).

Twenty-two normal controls were recruited from among the blood donors and staff of Siriraj Hospital.

Ethics

Approval for the study was granted by the Research Ethics Committee of the Faculty of Medicine, Siriraj Hospital. Written consent was obtained from each of the subjects and from the parents of the one pediatric patient.

Blood sample collection and lymphocyte preparation

Fifteen milliliters of peripheral venous blood were collected from each subject into a sterile tube containing EDTA-disodium salt as the anticoagulant. Peripheral blood mononuclear cells (PBMC) were then separated from the freshly collected whole venous blood by Ficoll-Hypaque density gradient centrifugation using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway).

RNA isolation

The total RNA was isolated from peripheral blood lymphocytes with a TRIZOL reagent (GIBCOBRL, Life Technologies Inc, Grand Island, NY, USA), used according to the manufacturer's instructions. RNA pellets were washed and then resuspended with 200 μl of 75% ethanol for storage at -70ºC until use.

cDNA synthesis

The cDNAs were prepared from *reg1α* and *reg1β* mRNA using SUPERSCRIPT™ II RT for First Strand cDNA Synthesis Kit (Life Technologies). An aliquot of total RNA (1-5 μg) was used for cDNA synthesis by mixing with a 0.5 mg oligo $(dT)_{15}$ primer and DEPC-treated water the aliquot was then incubated at 70ºC for 10 minutes before cooling on ice for 5 minutes. Thirty units of RNasin® Ribonuclease (Rnase) Inhibitor (Promega, Medison, USA) were then added to the mixture to protect the RNA. The reaction mixture was made up to 19 μl, containing 1 μl of 10x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mixture, 0.2 M dithiothreitol (DTT), and 200 U of SUPERSCRIPT™ II RT, which was added after all the other reagents had been incubated at 42ºC for 5 minutes. The cDNA synthesis was performed at 42ºC for 90 minutes and terminated by incubation at 70ºC for 15 minutes. 1 U of Rnase H (Life Technologies) was then added, and the reaction mixture was incubated at 37ºC for 20 minutes. An aliquot of the first strand cDNA was subsequently used for PCR. The remaining cDNA was kept at -20ºC until further use.

PCR and nested PCR

The PCR primers for the amplification of human *reg1α* and *reg1β* were designed by using the MacVector™ (version 4.5.1) program (Kodak Scientific Imaging Systems, Connecticut, USA). The mRNA sequences of the *reg1α* gene (accession number NM-002909) and the *reg1β* gene (accession number NM-006507) were retrieved from the Genbank database (http://www.ncbi.nlm.nih.gov). The primers were purchased through the Customer Synthesis Service of the BioService Unit, National Center for Genetic Engineering and Biotechnology (NCGEB), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

The cDNAs, constructed by using *reg1α* and *reg1β* mRNAs as templates, were amplified by PCR and nested PCR. For PCR, the reaction mixture contained 2.5 μl of 10x PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTP, 12.5 pmol each of L- and R-

primers, 0.25 U of *Taq* DNA polymerase, 2 μl of cDNA template, and 10.95 μl of sterile distilled water to make a total volume of 25 μl. Two drops of mineral oil were overlaid on the mixture. PCR was carried out for 40 cycles in a Thermal Cycler 480: each cycle consisted of denaturation at 95ºC for 5 minutes, annealing at 50ºC for 1 minute, and extension 72ºC for 1.5 minutes; a final extension step was carried out at 72ºC for 5 minutes.

For nested PCR, 1 μl of the primary PCR product was mixed in the total volume of 25 μl. The reagents and the PCR profile used in this step were the same as those used during the primary PCR.

Restriction endonuclease digestion of PCR product

Restriction sites of endonucleases on cDNA sequence were analyzed by the MacVector™ (4.5.1 program). The restriction endonuclease digestions were performed by mixing 10-20 μl aliquots of either the PCR product of *reg1α* with 2 U of *Eco*RI (New England Biolabs, USA) or the PCR product of *reg1β* with 2 U each of *Eco*RI and *Bam*HI (New England Biolabs, USA), 25 μl of buffer, 0.1 μg/ml of bovine serum albumin (BSA), and sterile water to a final volume of 25 μl. The reaction mixture was incubated at 37ºC for 5 hours. The restriction fragments were then resolved by electrophoresis on 2% agarose gel in TAE buffer with ethidium bromide staining.

Detection of single strand conformation polymorphism (SSCP) by electrophoresis and silver staining

A mixture of completely digested PCR products, obtained from restriction enzyme digestion of either *reg1α* or *reg1β* cDNA, was applied to 10% nondenaturing polyacrylamide gel. Electrophoresis was carried out in TBE buffer at room temperature and 20 mA constant current. The DNA band was detected by silver staining. Having been fixed in 50 ml of 40% methanol for 10 minutes, the gel was stained with 50 ml of 0.2% w/v $AgNO_3$ at room temperature, with continuous shaking for 20 minutes. Fifty milliliters of developer (3% w/v Na_2CO_3 and 0.0175% formaldehyde) were then added to the gel and left at room temperature for 4-10 minutes, with gentle shaking, until the stained DNA bands were revealed. The reaction was stopped by adding 70 ml of 10% w/v citric acid, and shaking for 5 minutes. The SSCP pattern was archived by scanning with a computer scanner (UMAX Data Systems, Taipei, Taiwan) loaded with an Adobe Photo Shop Program (version 4.0.1) (Adobe Systems Inc, Mountain View, USA).

Direct sequencing

The nested PCR products were purified from low-melting agarose gel by using a QIA Quick Gel Extraction Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Nested PCR sequences were determined by the ABI PRISM™ 310 Automated DNA sequencer (PE Applied Biosystems Inc, California, USA) using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc, California, USA) according to the manufacturer's instructions. Fluorescent signals were detected and saved with ABI collection software in a computer connected to the sequencer. The nucleotide sequence was determined by a Sequence Navigator and analyzed by Mac Vector software.

RESULTS

The entire coding regions of the *reg1α* and *reg1β* cDNAs were isolated from the peripheral blood lymphocytes obtained from 16 patients with fibrocalculous pancreatopathy, 42 patients with type 1 diabetes, 37 patients with type 2 diabetes, and 22 normal controls, using RT-PCR and nested PCR methods. The PCR products obtained had the expected sizes, *ie* 598 bp for *reg1α* and 662 bp for *reg1β*, as shown in Figs 1 and 2 respectively. Direct sequencing showed that the nucleotide sequences were the same as those in Genbank

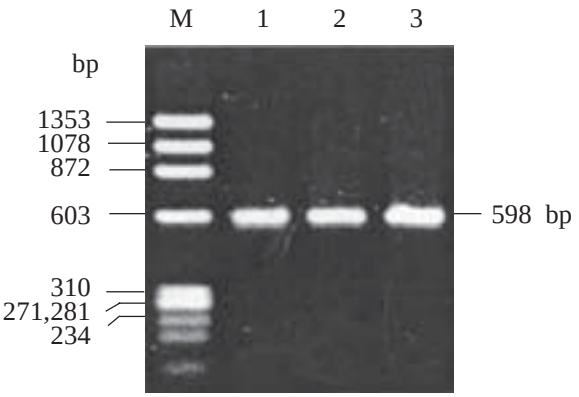

Fig 1–Agarose gel electrophoresis of the PCR products obtained from RT-PCR and nested PCR amplification of the *reg1α* mRNA coding region of human peripheral blood lymphocytes. The PCR sample was electrophoresed on 1% agarose gel in 1x TAE buffer. The size of the PCR products was 598 base pairs. Lane M is *Hae*III-digested øx174 DNA markers. PCR products and markers were stained by ethidium bromide.

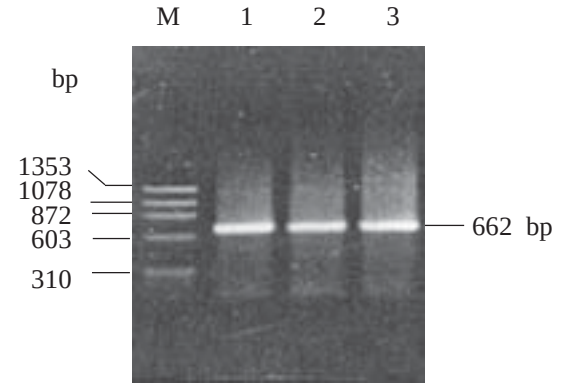

Fig 2–Agarose gel electrophoresis of the PCR products obtained from RT-PCR and nested PCR amplification of the *reg1β* mRNA coding region of human peripheral blood lymphocytes. The PCR sample was electrophoresed on 1% agarose gel in 1x TAE buffer. The size of the PCR products was 662 base pairs. Lane M is *Hae*III-digested øx174 DNA markers. PCR products and markers were stained by ethidium bromide.

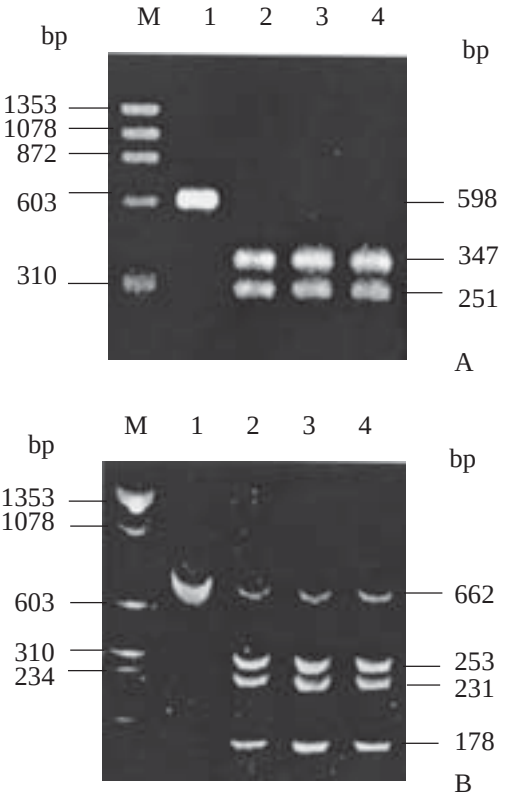

Fig 3–Agarose gel electrophoresis of the PCR products amplified from the coding region of *reg1α* (Fig 3A) and *reg1β* (Fig 3B). Lane 1 is the entire PCR product; lanes 2-4 are the restriction endonuclease-digested PCR products, *(Eco*RI for *reg1α* and *Eco*RI-*Bam*HI for *reg1β).* Lane M is *Hae*III-digested øx174 DNA markers.

(accession numbers NM-002909 and NM-006507).

After digestion of *reg1α* RT-PCR product by *Eco*RI, two fragments (one of 251 bp and the other of 347 bp) were obtained (Fig 3A). Three fragments (253 bp, 178 bp, 231 bp) were obtained from the digestion of the *reg1β*-RT PCR product by *Eco*RI-*Bam*HI (Fig 3B).

SSCP analysis of the enzyme-digested PCR products was performed and only one pattern was found for each gene in all the subjects studied (Fig 4). There was no mobility shift in the PCR-SSCP analysis: this indicated

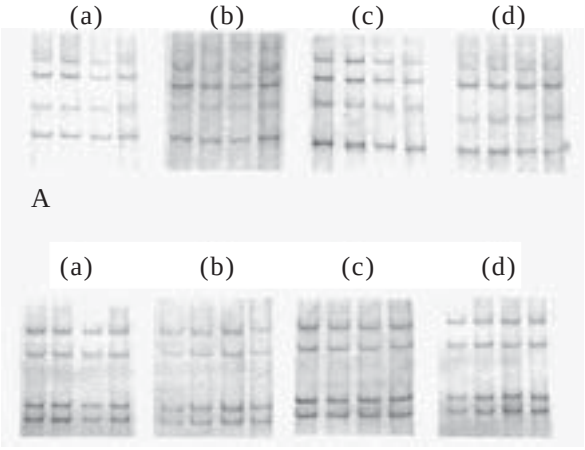

Fig 4–PCR-SSCP analysis of the enzyme-digested PCR products obtained from RT-PCR and nested PCR amplification of the coding region of *reg1α* (Fig 4A) and *reg1β* (Fig 4B) mRNA in peripheral blood lymphocytes, obtained from patients with type 1 diabetes mellitus (a), type 2 diabetes mellitus (b), fibrocalculous pancreatopathy/FCPD (c), and normal controls (d).

that there was no abnormality of the *reg1α* and *reg1β* RT-PCR products obtained from the 3 groups of diabetic patients and the normal controls. In addition, the sequencing results of *reg1α* and *reg1β* cDNAs from one patient with fibrocalculous pancreatopathy were no different to those of the normal controls.

DISCUSSION

Fibrocalculous pancreatopathy is a form of diabetes secondary to tropical chronic calcific pancreatitis, seen mostly in developing countries (Premalatha and Mohan, 1994; Mohan *et al*, 1998). Many studies have suggested an environmental etiology of the disease (Khan and Ali, 1997; Hassan *et al*, 2000). However, family studies have shown familial aggregation of the disease with evidence of the vertical transmission of fibrocalculous pancreatopathy from parents to their offspring. Several reports have demonstrated associations between fibrocalculous pancreatopathy and polymorphism of HLA-DQB 1 and the insulin gene. Fibrocalculous pancreatopathy is likely to be a multifactorial disease, with both genetic and environmental components (Mohan *et al*, 1989; Kambo *et al*, 1989).

It has been suggested that *reg* protein, which is encoded by the *reg1α* gene, is involved in the inhibition of pancreatic lithogenesis (Bernard *et al*, 1992); in addition, it has been found to be associated with islet β-cell regeneration and the amelioration of surgical diabetes in animals (Yonemura *et al*, 1984; Watanabe *et al*, 1994). Stone formation and β-cell destruction are evident in fibrocalculous pancreatopathy (Yajnik *et al*, 1992; 1997): it might be that a defect of *reg1* genomic DNA or its transcription causes these features of the disease.

We have recently studied *reg1α* and *reg1β* genomic DNA in diabetic patients: no abnormality of the genomic DNA associated with diabetes mellitus was demonstrated (Banchuin *et al*, 2002). Hawrami *et al* (1997) studied *reg1α* gene and found neither an abnormality of RFLPs nor SSCP variants of the gene obtained from patients with FCPD; moreover, the direct nucleotide sequencing of *reg1α* gene obtained from FCPD patients was no different to the published human *reg1α* gene sequence (Hawrami *et al*, 1997) – the abnormality of any gene can be at the transcription step, however.

In this study, we investigated the possibility of an abnormality of the mRNA transcription of the *reg1α* and *reg1β* genes. Because there was a limit to the amount of mRNA that could be readily obtained found in inaccessible cells, *eg* in the pancreas), the total RNA was obtained from peripheral blood lymphocytes. The amplification of the coding region of the *reg* genes, by RT-PCR and nested PCR, used two pairs of primers (outer and inner primers). The PCR products obtained had sizes and sequences which were the same as those that appeared in Genbank (accession numbers NM-002909 and NM-006507): this

indicated that there was an ectopic transcription of the *reg1α* and *reg1β* genes in peripheral blood lymphocytes, as found in *anti-Mullerian hormone, β-globin, aldolase A, factor VIIIC* and *PKD1* genes (Chelly *et al*, 1989; Thongnoppakhun *et al*, 1999; Akkarapatumwong *et al*, 2000). This ectopic expression of the *reg1α* and *reg1β* genes has not been reported before. This finding is in contrast to that of Watanabe *et al* (1990), which was that *reg* mRNA was not detected in lymphocytes, the liver, spleen, brain, thyroid gland, submandibular gland, esophageal mucosa and rectal mucosa, although it was detected at low level in the kidney. This discrepancy may be due to the very low amount of *reg1α* and *reg1β* mRNA in lymphocytes, which cannot be detected by Northern blot [the method used by Watanabe *et al* (1990)], but can be detected after amplification (as in our study). The results of our study have indicated that lymphocytes can be used as the source of mRNA for *reg1α* and *reg1β* mRNA analysis.

In this study, the entire coding regions of the *reg1α* and *reg1β* genes were isolated and amplified from their mRNA transcripts prepared from peripheral blood lymphocytes by RT-PCR and nested PCR. The amplified PCR products were then digested and screened for mutation by SSCP analysis. Only one pattern each was revealed for all the subjects studied, including patients with fibrocalculous pancreatopathy, type 1 and type 2 diabetes, and normal controls: this suggested that there was no abnormality of *reg1α* and *reg1β* transcription in the 3 groups of diabetic patients.

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