Do prion protein gene polymorphisms induce apoptosis in non-mammals?

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Genetic variations such as single nucleotide polymorphisms (SNPs) in prion protein coding gene, *Prnp*, greatly affect susceptibility to prion diseases in mammals. Here, the coding region of *Prnp* was screened for polymorphisms in red-eared turtle, *Trachemys scripta*. Four polymorphisms, L203V, N205I, V225A and M237V, were common in 15 out of 30 turtles; in one sample, three SNPs, L203V, N205I and M237V, and in the remaining 14 samples, only L203V and N205I polymorphisms, were investigated. Besides, C658T, C664T, C670A and C823A SNPs were silent mutations. To elucidate the relationship between the SNPs and apoptosis, TUNEL assays and active caspase-3 immunodetection techniques in brain sections of the polymorphic samples were performed. The results revealed that TUNEL-positive cells and active caspase-3-positive cells in the turtles with four polymorphisms were significantly increased compared with those of the turtles with two polymorphisms ($P<0.01$ and $P<0.05$, respectively). In conclusion, this study provides preliminary information about the possible relationship between SNPs within the *Prnp* locus and apoptosis in a non-mammalian species, *Trachemys scripta*, in which prion disease has never been reported.

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1. Introduction

Prion diseases, also referred as transmissible spongiform encephalopathies (TSEs), are a group of progressive neurodegenerative pathologies that include kuru, Creutzfeldt-Jakob disease (CJD) and familial fatal insomnia (FFI) in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. These disorders widely affect humans and animals due to their acquired (<1%), genetic (~15%) and sporadic (~85%) nature (Pastore and Zagari 2007; Shkundina and Ter-Avanesyan 2007, Soto and Estrada 2008; Moore *et al*. 2009; Makarava *et al*. 2011). The hallmark of prion diseases is aggregation of misfolded pathogen isoform (PrP^{Sc}) of cellular (PrP^{C}) prion protein in central nervous system (CNS). Although PrP^{C} and PrP^{Sc} are derived from same mRNA and indicate an amino acid homology, PrP^{Sc} has different biochemical and structural properties such as increased beta-strand content and resistance to proteinase K (Prusiner 1998). According to the 'protein-only hypothesis' replication of the PrP^{Sc}, the disease-related isoform, involves changing the conformation of normal cellular precursor, PrP^{C} (Watts and Westaway 2007). For further isoforms, PrP^{Sc} acts as a conformational template and promotes the $PrP^{C} \rightarrow PrP^{Sc}$ conversion (Cappai and Collins 2004). The normal prion protein (PrP) is a cell surface glycoprotein that is attached to cell membrane by means of glycosylphosphoinositol (GPI) anchor, but it can also accumulate in other cellular sites such as Golgi apparatus, endosomes, and in cytoplasm (Fournier *et al*. 2000; Laine *et al*. 2001; Mironov *et al*. 2003). It is widely expressed in central nervous system (CNS), particularly in neurons. PrP protein encoding gene (*Prnp*) (Chesebro *et al*. 1985; Oesch *et al*. 1985; Basler *et al*. 1986) has been characterized in mammalian (Rivera-Milla *et al*. 2003; van Rheede *et al*. 2003) and non-mammalian species (Gabriel *et al*. 1992; Calzolai *et al*. 2005, Malaga-Trillo *et al*. 2011) and in yeast (Li *et al*. 2004). Although

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little is known about the physiological roles of PrP, a wide array of cellular functions have been attributed to PrP^C including involvement in signal transduction, cell adhesion, regulation of immune system, neurite outgrowth, oxidative stress and cell death and survival (Zomosa-Signoret *et al*. 2008). Some of the genetic variations can alter the amino acid sequence of the protein structure, and thereby they can affect the corresponding protein function (Ng and Henikoff 2006). Polymorphisms within the *Prnp* appear to constitute a part of the genetic background for susceptibility or resistance of prion diseases by inducing structural changes within the protein motifs (Westaway *et al*. 1987; Prusiner and Scott 1997; Tranulis 2002; Baylis and Goldmann 2004; Mead 2006). For instance, a single nucleotide polymorphism (SNP) at codon 129 (M>V) is known to confer susceptibility to sporadic CJD (Palmer *et al*. 1991; Jeong *et al*. 2005), and this polymorphism is an important factor for human prion diseases due to its modulator effects on the molecular processes of prion propagation (Hosszu *et al*. 2004; Lewis *et al*. 2006). Other *Prnp* polymorphisms in humans are G127V, G142S, N171S, V180I, E219K and M232R (reviewed in Lloyd *et al*. 2013). In addition to effect on prion propagation, polymorphisms are also associated with 'species barrier', a concept explain that inadequate transmission between the mammalian species (Hill and Collinge 2002; Weissmann 2005). In general, cross-species transmission of the TSE agent is less efficient than inter-species transmission due to species barrier (Hagiwara *et al.* 2013).

The role of PrP^C on cell death is conflicting. Although the observations that denote overexpression of PrP^C causes caspase-3- and/or mitochondria-mediated apoptosis (Paitel *et al*. 2002; Hachiya *et al*. 2005; Wang *et al*. 2009), a series of distinctively conducted experiments demonstrated that PrP^C protects neurons against Bax-mediated apoptosis due to similarity in amino acid sequences between octapeptide repeats of PrP and BH-2 domain of Bcl-2 family members (Bounhar *et al*. 2001; Roucou *et al*. 2005). Moreover, its protective role against staurosporine-mediated cellular stress has also been investigated (Zhang *et al*. 2006). There is no doubt that the mechanism underlying this dilemma highly fascinating and still remains largely mysterious. As mentioned above, the current state of the literature supports a prominent role for prions in negative control of apoptosis. To our knowledge, the association between apoptosis and genetic polymorphisms within the *Prnp* is poor understood. Therefore, in the present study we aimed to search the effect of SNPs within *Prnp* to apoptosis in a non-mammalian model, red-eared turtle, *Trachemys scripta.* The evolutionary origin of PrP^C is still unclear and hence, it is uncertain as to whether *Trachemys scripta* is affected by TSE. Because of the studies on non-mammalian species such as reptiles may give us an opportunity to understanding the evolutional and functional properties of PrP and of prion transmission across species barriers, we performed a comparative analysis of the sequence of the prions between *Trachemys scripta* and human, as well as preliminary evaluation of how can the polymorphism levels in the prion affect the cell apoptosis.

2. Materials and methods

2.1 *Ethics approval*

This study was approved by the Animal Experimentation Ethics Committee of the Ege University, Izmir, Turkey (Approval number: 2009-12). All experiments were made to minimize animal suffering and limit the number of animals used (n=30).

2.2 *Animals and tissue preparation*

Turtles were purchased from Concordia Turtle Farm, New Orleans, LA, USA. In order to obtain liver and brain tissues, turtles were sedated with ketamine-xylasine mix, and dissected. Liver tissues were stored at +4°C for further DNA isolation, and brain tissues were fixed with 10% formalin for Haematoxylin-Eosin (H&E), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, caspase-3 immunohistochemistry (IHC) and caspase-3 immunofluorescence (IHC-F).

2.3 *DNA isolation, amplification and sequencing*

For the molecular analyses, liver tissues were used. The brain tissues, which were very small, were used for immunohistochemical, immunofluorescence, and immunolabeling assays as well as H&E staining. Genomic DNA was isolated from liver samples using High Pure PCR Template Preparation Kit (Roche, Cat # 11 796 828 001) according to the manufacturer's instructions. *Prnp* amplification performed by polymerase chain reaction (PCR) using the following forward and reverse primers corresponding to turtle *Prnp* nucleotides 96-459 and 436-908, respectively:

(1) 5′-TTG TGA TGT GGA GCG ACG TTT CCT-3′ and
(2) 5′-AAC CAA CAT GAA AGC CAT GGC AGG-3′ (forward)

(1) 5′-CCT GCC ATG GCT TTC ATG TTG GTT-3′
(2) 5′-GTT TGC ACA GAT CAG CAG CAC AGT-3′ (reverse) (Genbank access number: AJ245488.1).

PCRs were carried out in a 25 µL reaction volume containing 50–100 ng genomic DNA, 1 U Taq polymerase, 200mM dNTPs, 5µM of each primers and 1.5 mM $MgCl_2$. Amplifications were performed in a Amplitronyx™ 6 Thermal Cycler (Nyx Technik Inc., CA, USA) through a heat step at 94°C for 2 min, 35 cycles at 94°C for 1 min, primer

binding step at 56°C for 1 min, and finally, an extension step at 72°C for 1 min. All PCR products were visualized for yield and purity following a 2% agarose gel electrophoresis and ethidium bromide staining (not shown). The PCR products were purified with GF-1 PCR Clean-Up Kit (Vivantis, Cat # GF-PC-050). Sequencing of prepared DNA fragments was performed with a sequence analyser at Iontek DNA Sequencing Facility (İstanbul, Turkey).

2.4 *Polymorphic analysis of Prnp*

The following software and tools were conducted: ChromasPro® for sequence analyses (*http://technelysium.com.au*), BLAST (Basic Local Alignment Search Tool) for sequence verification (*www.ncbi.nlm.nih.gov/BLAST/*), Translate tool of ExPASy (*www.expasy.org*) for DNA to protein translation and ClustalW2 software for sequence alignment (*http://www.ebi.ac.uk/Tools/msa/clustalw2/*).

2.5 *Cell death assays*

To identify and quantify apoptotic cells in brain tissues of polymorphic samples, colorimetric (TUNEL) and flourometric [TUNEL-fluorescence (TUNEL-F)] assays were performed using *In Situ* Cell Death Detection Kit, POD (Roche Applied Science, Cat # 11684817910) following manufacturer's protocol with slight modifications. Briefly, dewaxed and rehydrated sections were permeabilized with proteinase K at 37°C for 15 min and washed with phosphate buffered saline (PBS). The sections were incubated with TUNEL reaction mix (labelling solution and TdT enzyme) at 37°C for 1 h. For negative control, only labeling solution was used. After incubation, for colorimetric evaluations converter-POD was added to each samples and incubated at 37°C for 30 min. Samples were treated with 3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB) until the color reaction was seen and 0.5% methyl green was added as counterstain. Subsequently, the sections mounted with a non-aqueous medium (Vectamount®, Vector Laboratories, Cat # H-5000) and photographed under bright field microscopy (Olympus® BX43, Tokyo, Japan). For fluorescent microscopy, similar steps were conducted except the converter-POD incubation. All of the micrographs and flourographs were captured as JPEG format (resolution of 2272×1704 pixels) at a magnification ×100 using Olympus® BX43 microscope (Tokyo, Japan) and Nikon® Coolpix E4500 digital camera system (Tokyo, Japan). The TUNEL-positive cells were counted in randomly selected areas by using 'cell counter' plugin of the ImageJ software, version 1.46c (NIH, Bethesda, MD, USA; *http://rsb.info.nih.gov/ij/*), and the 'apoptotic index' (AI) calculated as follows:

AI(%) : the number of positively stained apoptotic cells/total number of cells counted × 100%.

Finally, the results were scored as low (samples with >15% AI), positive (15–50% AI) and high (51–100% AI).

2.6 *Caspase-3 immunohistochemistry and immunofluorescence*

For caspase-3 immunohistochemistry (Casp-3 IHC) and immunofluorescence (Casp-3 IHC-F), paraffin embedded cross-sections of optic tectum, 5 µm thick, dewaxed and rehydrated were used. For IHC, sections underwent heat-induced antigen retrieval in 10 mM citrate buffer, pH 6.0 for 20 min in a water bath and cooled to room temperature for 20 min. After extensive washes in PBS, endogenous peroxidase activity was quenched by incubation for 10 min at room temperature with 3% solution of H_2O_2 in methanol. Following by a blocking step (IHC Blocking Reagent, Millipore, Cat # 20773) for 30 min at 37°C, the sections were immunostained overnight at +4 °C with primary antibody for active caspase-3 (1:200 dilution, Abcam, Cat # ab13847). The sections were subsequently incubated with biotinylated secondary antibody (Goat anti-Rabbit IgG-H&L, 1:400 dilution, Abcam, Cat # ab6720) for 1 h at room temperature in a humid chamber, washed in PBS. For bright field light microscopy, the sections were incubated with biotinylated streptavidin peroxidase (Vector Laboratories, Cat # SA-5004) for 30 min at same conditions. Immunoreactivity was visualized with DAB (Vector Laboratories, Cat # SK-4100) as a chromogen. The slides were counterstained with methyl green and coverslipped with Vectamount® (Vector Laboratories, Cat # H-5000). Positive reaction was identified as a brownish color. For immunofluorescence, sections were incubated overnight at +4°C with primary antibody for active caspase-3 (Abcam, Cat # ab13847) in incubation buffer (1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton X-100 in PBS, 1:100 dilution). After removing the primary antibody with several washes in PBS, the sections were incubated for 1 h at room temperature in a humid chamber with fluorescein isothiocyanate (FITC) conjugated secondary antibody (Goat Anti-Rabbit IgG-FITC, 1:400 dilution, Santa Cruz, Cat # sc-2012) in incubation buffer. Finally, as a nuclear counterstain, 4′6-diamidino-2-phenylindole (DAPI) (Santa Cruz, Cat # sc3598, 1:1000 dilution in McIlvaine's buffer) was added for 3 min before sections mounted (Ultracruz Hard-set mounting medium, Santa Cruz, Cat # 359850). Sections with primary antibody omitted were

used as the negative controls. All immunohistochemical and immunofluorescent slides were examined by Olympus® BX43 microscope (Tokyo, Japan). All digital images were captured as JPEG format (resolution of 2272×1704 pixels) using Nikon® Coolpix E4500 digital camera system (Tokyo, Japan). Expression of caspase-3 positive cells was evaluated in randomly selected visual fields by bright field light and fluorescence microscopy (Olympus BX43, Tokyo, Japan) at 100× magnification and counted with ImageJ software. Quantitatively, percentage of caspase-3-positivity (the number of positively stained cells/total number of cells counted × 100%) was scored by a simplified immunoreactivity score (IRS) without considering the staining intensity as follows: low (samples with > 15% caspase-3-positive cells), positive (15–50% caspase-3-positive cells) and high (51–100% caspase-3-positive cells).

2.7 *Statistical analysis*

All the data were statistically analysed using IBM SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was performed for compare the AI and caspase-3 positivity between two and four SNP containing samples. All data are expressed as mean ± SEM. A *P*-value less than 0.05 were deemed to indicate statistical significance.

3. Results and discussion

3.1 *DNA sequence analyses*

We found eight SNPs, four (C658T, C664T, C670A and C823A) silent mutations and four (L203V, N205I, V225A, M237V) that promote an amino acid change in the open reading frame (ORF) of the *Trachemys scripta Prnp* gene. Two of these polymorphisms (L203V and N205I) were common in all turtles. Fourteen out of the 30 turtles were carry only two polymorphisms (L203V and N205I) and in 15 turtles four polymorphisms (L203V, N205I, V225A, M237V) were coexisted. In addition, only one sample was showed three SNPs (L203V, N205I, M237V) (table 1).

The predicted sequence of *Trachemys scripta* PrP was 270 amino acids in length that contain evolutionary conserved regions such as a signal peptide (residues 1–30), a long repeated region (residues 43–99) and secondary structural elements β1 (residues 151,152), α1 (residues 166–174), β2 (residues 184, 185) α2 (residues 197–209) and α3 (residues 222–246) strands with a C-terminal GPI signal sequence (residues 249–270).

Prion protein is highly conserved in mammals (Wopfner *et al*. 1999; Rivera-Milla *et al*. 2006). In the current study and as previously reported (Simonic *et al*. 2000), the amino acid sequence identity between human and Trachemys was about 38%. Human PrP and other mammalian species contain an octapeptide repeat region that is responsible for modulation of PrP aggregation through Cu^{+2} binding (Yu *et al*. 2008). However, the corresponding region in Trachemys PrP, consists of heterogeneously repeated ten hexapeptide sequences (PXYPXN-or G), was considerable different in size and structure from those of human and other mammalian species. The histidine residues, that actually bind the Cu^{+2}, were lack in Trachemys hexapeptide repeat region. It contains only two non-flanking histidine amino acids. It is thus conceivable that hexapeptide repeats in Trachemys PrP remain incapable for the PrP aggregation due to absence of Cu^{+2} binding capacity. KKRPKP, 23rd–28th residues in human, is a glycosaminoglycan binding motif and play an essential role PrP endocytosis, (Sunyach *et al*. 2003; Taylor *et al*. 2005; Yu *et al*. 2008). This segment was strongly deviated in Trachemys with the substitutions in the third (G), fourth (K), fifth (G) and seventh (G) positions and K homologies in first, second and sixth positions (KKGKGKG-residues 25–32). Nevertheless, the AGAAAAGA sequence, known as the responsible for the generation of PrP^{C}- PrP^{Sc} complex and prion propagation (Norstrom and Mastrianni 2005), was retained similar in Trachemys (figure 1A).

Table 1. Analysed polymorphisms of *Trachemys scripta Prnp*

Nucleotide position	Codon position	Allele	Codon	Polymorphism
658	202	C > T	TG**C** → TG**T**	Silent
659, 661	203	C > G, C > G	**C**T**C** → G**T**G	L > V
664	204	C > T	AA**C** → AA**T**	Silent
666	205	A > T	A**A**C → A**T**C	N > I
670	206	C > A	AC**C** → AC**A**	Silent
726	225	T > C	G**T**C → G**C**C	V > A
761	237	A > G	**A**TG → **G**TG	M > V
823	257	C > A	CT**C** → CT**A**	Silent

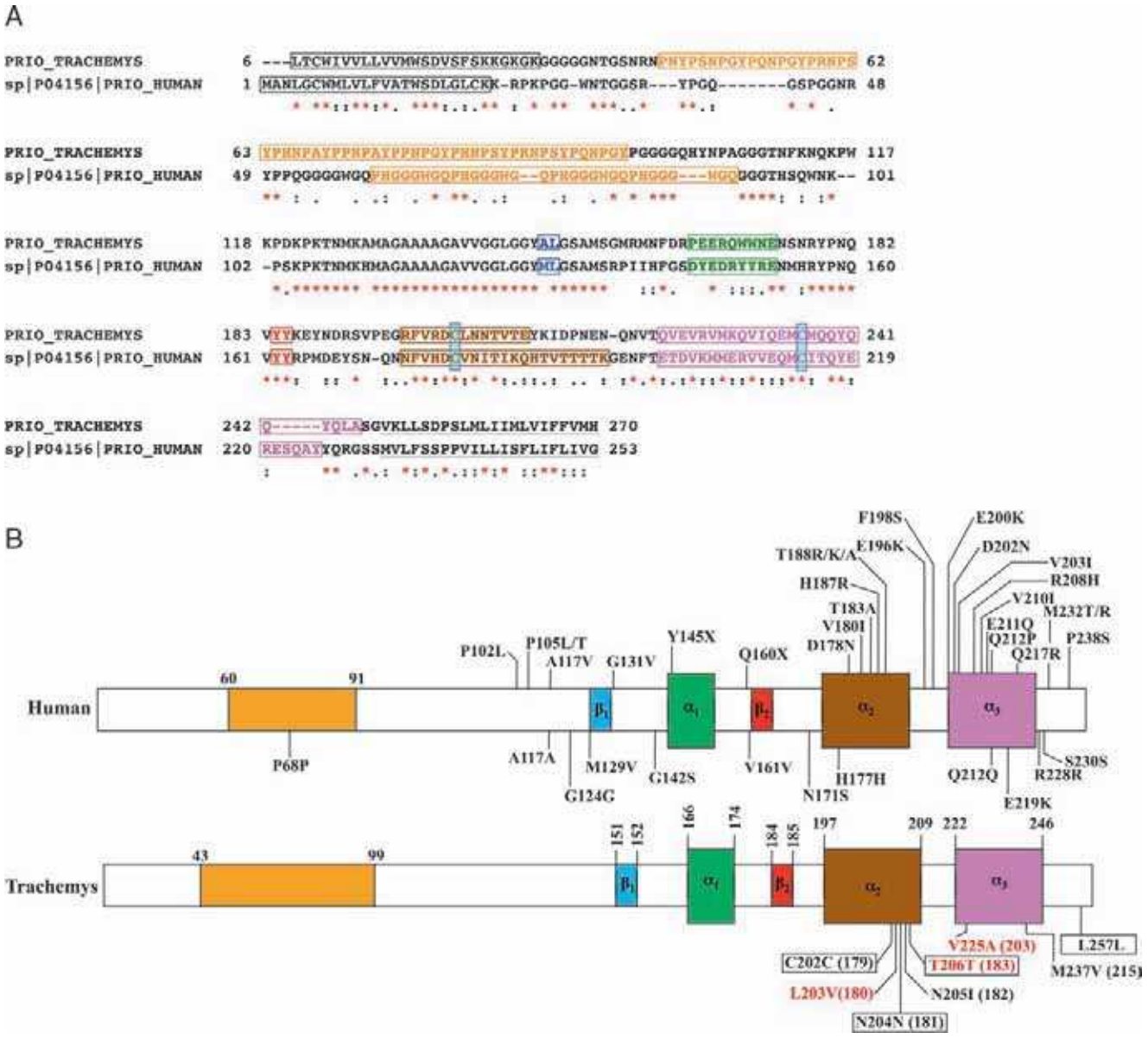

Figure 1. Sequence alignment of human and Trachemys (**A**) and schematic representation of the domain structure of the human PrP (upper panel) and the Trachemys PrP (bottom panel) and comparison of SNPs between human and Trachemys (**B**). Deduced amino acid sequence of Trachemys PrP was aligned with that of human PrP (accession number P04156) that is collected from The Universal Protein Resource (UniProt) (*http://www.uniprot.org/uniprot/*) using with ClustalW2 software from The European Bioinformatics Institute (*http://www.ebi.ac.uk*). Identical amino acids were marked in a red asterisk; double dots (:) represent a conserved residue substitution; a single dot (·) shows partial conservation of the residue. Coloured letters and boxes indicate regular secondary structural elements of the protein. The putative signal sequence is identified by a black box. In contrast to octapeptide repeats of human PrP, Trachemys include 10 hexapeptide repeats sequencing as PXYPXN/G (orange box). In addition, hexapeptide sequences are deprived of Cu^{+2} binding histine residues (see text). Blue, green and red boxes indicate β1, α1 and β2 domains, respectively. The structures of β2 strand were identical. However, the α2 sequence of human PrP was slightly longer than that of the Trachemys (brown boxes). The cysteine residues potentially involved in the formation of intramolecular disulfide bonds (S-S) are highlighted with light blue boxes in α2 and α3 (magenta boxes) strands (residues 179 and 214 in human, 202 and 214 in Trachemys). Underlined amino acids indicate the C-terminal GPI-signal sequences (**A**). Polymorphisms in human and Trachemys have been compared in panel B. Upper lane indicates *Prnp* SNPs that cause human prion diseases (Colby and Prusiner, 2011; Mead, 2006). At the bottom line, polymorphisms with silent mutations (black boxes) found in Trachemys were shown. Overlapping SNPs were identified by red letters. Brackets represent corresponding codon numbers in human (**B**).

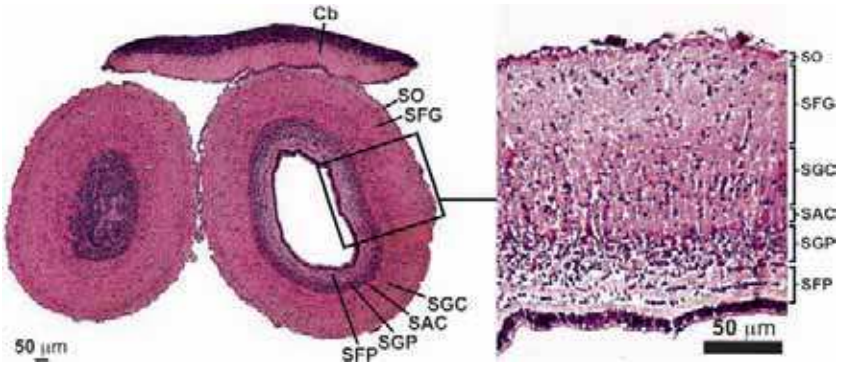

Figure 2. Parts of optic tectum in *Trachemys scripta*. SO; Stratum opticum, SFG; Stratum griseum et fibrosum superficiale, SGC; Stratum griseum centrale, SAC; Stratum album centrale SGP; Stratum griseum periventriculare, SFP Stratum fibrosum periventriculare. Cb; cerebellum. H&E staining. Bars indicate 50 µm.

The β1 region containing sequence of GYMLG (residues 127–131 in humans and 149–153 in Trachemys) is highly conserved in mammals (Nystrom and Hammarstrom 2014) whereas it slightly deviated in position 3 (codon 151) in Trachemys PrP. This variation coincides with M > V polymorphism at codon 129 that is highly relevant with CJD susceptibility in humans (Goldfarb *et al*. 1992). Intriguingly, SNPs at codons L203V, V225A and silence mutation at codon T206T in Trachemys were coincided with V180I, V203I and T183A conversions that are related with human prion diseases such as familial CJD (Mutsukura *et al*. 2009; Yang *et al*. 2010; Iwasaki *et al*. 2011), CJD (Peoc'h *et al*. 2000) and dementia (Grasbon-Frodl *et al*. 2004), respectively (figure 1B). Previously it has been indicated that the methionine residue at codon 129 of the human PrP sequence have a redox potential that is associated with oxidative modification of protein and hence it might be important in prion diseases (Barnham *et al*. 2006). Meanwhile, non-mammalian species possessing variant amino acids at this position are not associated with disease-related redox events (Ji *et al*. 2007). Therefore, we can't associate our overlapping findings with prion disease in Trachemys. However, these polymorphic coincidences and other divergences that were mentioned above may be important to gain an evolutionary perspective on the understanding of physiological roles of prion protein in non-mammalian species such as *Trachemys scripta*.

3.2 *Analysis of cell death*

In reptiles, optic tectum is composed of mainly six regions (figure 2) (Huber and Crosby 1933). To investigate whether SNP quantity on PrP had any effect on apoptotic response in optic tectum, turtles were clustered into two groups on the basis of their polymorphism intensities: the group of two polymorphisms (L203V, N205I, n=14) and the group of four polymorphisms (L203V, N205I, V225A, M237V, n=15). Apoptotic activity was evaluated by colorimetric and fluorometric assessments such as TUNEL assay and immunodetection of active caspase-3. We observed that TUNEL and caspase-3 staining colocalized among the superficial and the central regions, whereas there were only a few apoptotic cells in the periventricular region in both groups (figure 3A).

Optic tectum, which is homologue to the superior colliculus in mammals (Gandhi and Katnani 2011), is a part of the central nervous system of turtles (Kenigfest and Belekhova 2012), and hence the findings on optic tectum may be involved in other areas of turtle brain. In addition, the neurochemical organization of reptiles is similar to that of the avian species (Kenigfest and Belekhova 2012). So far, to our knowledge, there is no data on the evaluation of the connection between SNPs and neural cell death in CNS not only in non-mammals but also in mammalian species. However, in some experimental models and in transgenic mice it has been found that injection of prion disease agents can induce PrP accumulation and neuronal cell death induction in superior colliculus (Russelakis-Carneiro *et al.* 2004; Capobianco *et al.* 2007). It is important note that although the turtles were not infected with a prion disease agents due to species-barrier, our findings showed optic tectum is an essential involvement site for apoptosis as seen in its mammalian homologue, superior colliculus (Capobianco *et al.* 2007). On the other hand, the most intriguing finding was the increased number of apoptotic cells in the group of four polymorphisms when compared to that of the group of two polymorphisms ($P<0.01$; $P<0.05$) (table 2; figure 3B) in the optic tectum. Likewise, previous works suggest that neuronal cell death in the brain regions is highly related with PrP genotype, strain of prion and inoculation site in experimental prion diseases (Jesionek-Kupnicka *et al.* 2001). However, whether the SNP positions and/or amino acid substitutions

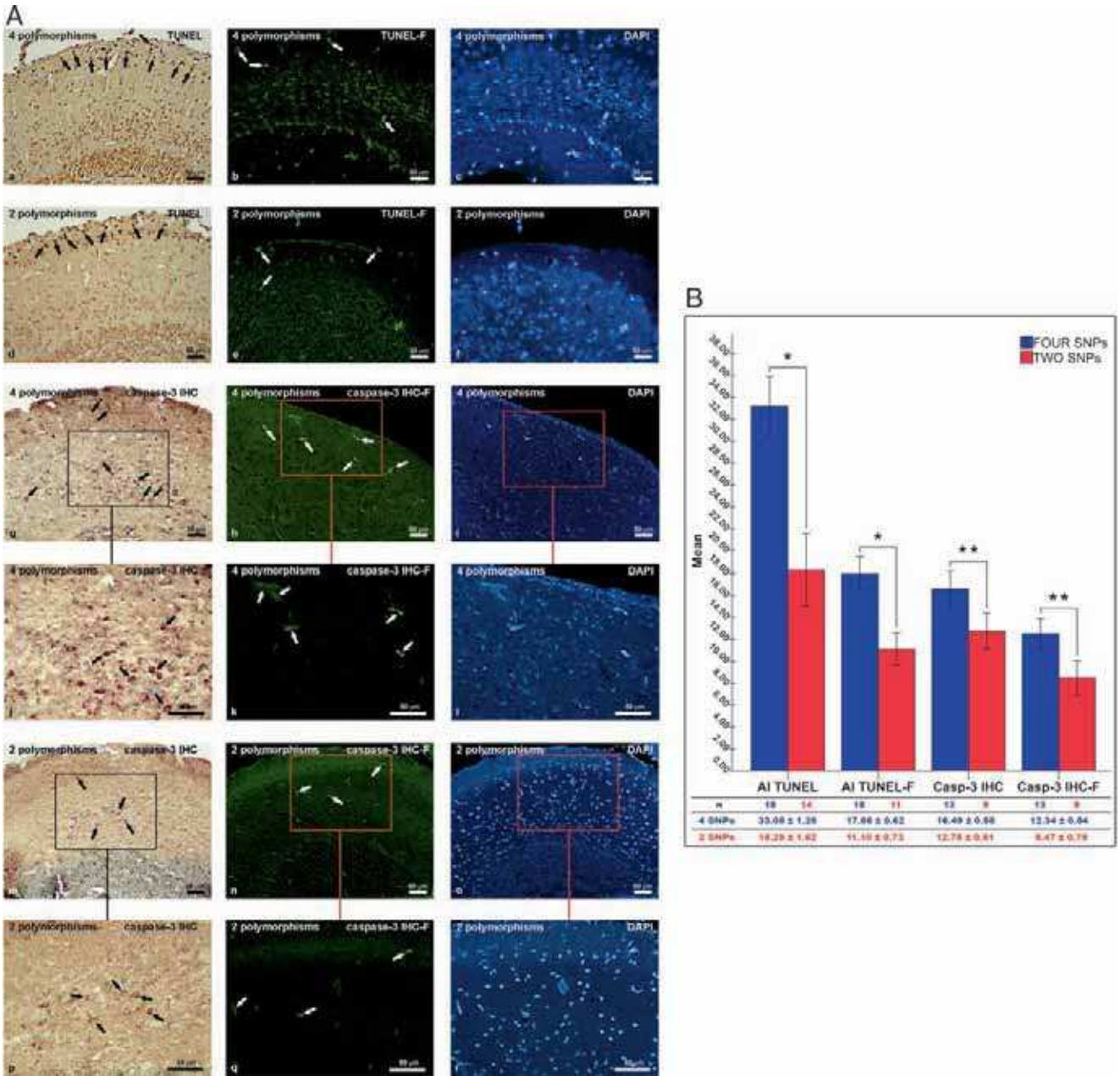

Figure 3. Representative pictures of TUNEL and caspase-3 IHC/IHC-F assays in turtles with four polymorphisms and turtles with two polymorphisms. DAPI staining was performed as nuclear marker (**A**). Turtles with two polymorphisms showed significant reduction in TUNEL-positive cells compare with turtles with four polymorphisms ($P<0.01$) (a, d; marked by black arrows). Immunofluorescence labeling confirmed this observation ($P<0.01$) (b, e; marked by white arrows). DAPI staining shows nuclei (c, f). In addition, the relative expression of active caspase-3 was determined by immunohistochemical and immunofluorescence techniques. Consistent with TUNEL results, caspase-3 activity significantly decreased in turtles with two polymorphisms when compared the turtles with four polymorphisms with caspase-3 IHC ($P<0.05$) and caspase-3 IHC-F ($P<0.05$). Arrows point to caspase-3 positive cells (g, h, j, k, m, n, p, q). DAPI staining serves as nuclear staining (i, l, o, r). It can be seen from the images that both the expression of caspase-3 and TUNEL-positive cells, and hence the levels of apoptotic cells were markedly different in each group. (**B**) The histograms showed apoptotic index and expression levels of caspase-3-positive cells which are quantified by ImageJ (see Materials and Methods) (n, points the number of randomly selected regions for the quantification). Values are the means ± SEMs (bottom line). $*P < 0.01$; $**P < 0.05$.

Table 2. Distribution of TUNEL-positive and caspase-3-positive cells in the group of four polymorphisms and the group of two polymorphisms

	Apoptotic Index						Caspase-3					
	TUNEL			TUNEL-F			Casp-3 IHC			Casp-3 IHC-F		
	Low	Positive	High	Low	Positive	High	Low	Positive	High	Low	Positive	High
Group of Four polymorphisms	-	15	-		15	-	4	9	-	13	-	-
Group of two polymorphisms	5	9	-	10	1	-	8	1	-	9	-	-

elicit cell death is largely unclear in Trachemys PrP. Although some genetic variations, like polymorphisms, do not induce diseases by themselves (Acevedo-Morantes and Wille 2014), they may influence the susceptibility to prion diseases (Prusiner 2001). At codon 129, for instance, neither V nor M substitution seems to be pathogenic on their own (Araujo 2013), but they are susceptible factor for prion diseases (Mead *et al.* 2009). However, people with the D178N polymorphism who are homozygous for V129 appear to get CJD (Lloyd *et al*. 2011). Consequently, it can be said that polymorphic combinations most likely orchestrate the prion structure and thereby functions of protein in both physiological and/or pathological states. Recent studies indicated that abnormal accumulation of cytosolic prion could disrupt the cytoskeleton and hence trigger an apoptotic pathway via Bcl-2 phosphorylation (Asnaghi *et al*. 2004, Li *et al*. 2011). Moreover, PrP mutants containing four and seven extra octapeptide repeats can cause ER stress (Xu *et al*. 2011). On the other hand, caspase-3 has been shown to cleave Bcl-2 to a Bax-like peptide (Cheng *et al*. 1997). Caspase activation has been observed in brain tissues of human CJD patients (Puig and Ferrer 2001) and mice in prion disease (Lawson *et al*. 2010). We showed that, caspase-3 activity significantly increased in brain tissues of turtles with four SNPs (figure 3). This observation suggests that polymorphisms in PrP sequence might induce apoptosis through caspase activation and Bax-mediated downstream events. This hypothesis can be supported by protective effect of non-polymorphic cellular PrP against apoptosis. Most recently Laroche-Pierre and her colleagues have reported that α3-helix of cytoplasmic PrP has an anti-Bax effect and the substitution of K204, V210 and E219 by proline inhibits the anti-Bax property of PrP (Laroche-Pierre *et al*. 2009). Similarly, the polymorphisms of α2-helix D178N and T183A inhibit the anti-apoptotic effect of PrP (Bounhar *et al*. 2001). Consistent with these reports, we observed that L203V, N205I, V225A, M237V SNPs in PrP more apoptotic than L203V, N205I SNPs.

We focused on two possible scenarios underlying increased apoposis in turtles with four polymorphisms: Firstly, increased polymorphisms can affect the protein folding and stability of PrP and hence decrease the anti-Bax activity. Intriguingly, V225A and M237V SNPs, which are located in α3-helix region of Trachemys PrP, may be key genetic variations that affect the protein folding and stability responsible for the caspase-3 activation and Bax-induced apoptosis (data not shown). In our opinion, to evaluate this scenario the level of Bax proteins should be compared. The second scenario is related with the accumulation of polymorphic proteins in cytosol. This hypothetical scenario may be related with other cell death types, like autophagy and/or anoikis. It is well known that misfolded proteins are the targets of

endoplasmic reticulum-associated degradation (ERAD) system. Some of mutant proteins were aggregated in endoplasmic reticulum membrane and degraded by auotophagic machinery (Lu *et al.* 2003; Kaushal 2006; Fujita *et al.* 2007; Jeong *et al.* 2012, 2013). Indeed, recent studies discussed the role of autophagy in TSEs (Liberski *et al.* 2008; Yao *et al.* 2013). Anoikis is a cell death type induced upon cell detachment from extracellular matrix and other cells. The caspase-mediated cleavage of focal adhesion kinase (FAK) (Alahari *et al.* 2002), disruption of the cytoskeleton (Puthalakath *et al.* 1999) and blockage of E-cadherin molecules (Bergin *et al.* 2000) can induce anoikis. Previous works show that one of the physiological functions of PrP is regulation of the stability of E-cadherin/beta-catenin complex (Malaga-Trillo *et al.* 2009). However, it has been reported that cytosolic PrP accumulation is not related apoptosis (Mironov *et al.* 2003). Therefore, detailed analyses of E-cadherin and other anoikis markers such as actin and β-catenin may be important to evaluate the second scenario.

In conclusion, in the present work, SNPs of prion protein coding gene in a non-mammalian model were examined and the quantity of SNPs was evaluated for apoptotic index for the first time. TUNEL and caspase-3 analyses showed that turtles with two polymorphisms undergo apoptosis more than turtles with two polymorphisms. Although the general view of prion diseases are precluded by non-mammals (Rivera-Milla *et al.* 2003; Ji *et al.* 2007), further analyses on structure-function of PrP in non-mammals can provide us new and important data on the human prion diseases. Therefore, *Trachemys scripta* may be offered as an essential model for prion biology in further works with this animal.

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