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Effects of hypoxia on the mRNA expressions of TRAIL-mediated cell death related genes in hypoxia-tolerant rodent (*Nannospalax nehringi*) and some characteristics of these proteins

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Abstract: The aim of the present study was to determine the mRNA expression profiles of TRAIL-mediated cell death related genes of the hypoxia-tolerant rodent *Nannospalax nehringi* under hypoxia. The nucleotide and amino acid sequences of these genes were identified. Captured *Nannospalax nehringi* were randomly divided into normoxia and hypoxia groups. The hypoxia group was exposed to a 7% O_2 + 93% N_2 gas mixture for 52 h, while animals in the normoxia group were housed under normoxic conditions. Total RNAs were isolated from brain and lung tissues. The cIAP-1, cIAP-2, XIAP, DcR1, DcR2, FLIP and OPG genes were PCR amplified, and the cIAP-1, cIAP-2, XIAP, OPG, TRAIL and FLIP genes were sequenced. Sequenced genes were translated into amino acid sequences and compared with reliable sequences of closely similar species. The genes in the brain were regulated for protection against hypoxia; however, the genes in the lung were regulated differently. Many mutations and insertions were observed on the conserved regions of the cIAP-1, cIAP-2, XIAP, OPG and TRAIL genes in *N. nehringi*. We propose that these gene mutations and insertions contribute to the anti-hypoxic properties of *N. nehringi*.

Keywords: apoptosis; *Nannospalax nehringi*; hypoxia; TNF-related apoptosis-inducing ligand (TRAIL); inhibitors of apoptosis (IAPs); FLICE-like inhibitory protein (FLIP)

INTRODUCTION

Apoptosis or programmed cell death, which is observed in both multicellular and unicellular organisms, has multiple complex steps [1]. The occurrence of apoptosis in different animal taxa reveals the importance of protecting the generation line in unicellular organisms and also preserving the continuation of physiological pathways in multicellular organisms. Apoptosis is an effective way to kill cancer cells. Therefore, abolishing cancer cells by apoptosis via cytotoxic agents is an effective way to deal with cancer cells, with minimal effects on non-cancerous cells. However, mutations that affect the apoptotic signaling pathways in cells can lead to apoptotic resistance.

The systemic use of most ligands of the tumor necrosis factor superfamily (TNFSF) members is highly cytotoxic to normal cells, except for the TNF-related apoptosis inducing ligand (TRAIL). The TRAIL induces apoptosis by selectively targeting cancer cells [2], and it is distinguished from other TNFSF member ligands by two important properties: it has no side effects in systemic use [3], and it can accomplish programmed cell death through a p53-independent pathway, which is mostly mutated in terminal cancers [4]. TRAIL is also effective in Type-I cells where caspase-8 can directly transmit apoptotic signals, and in Type-II cells in which the activation of proapoptotic proteins of mitochondria is essential, because it is capable of transmitting apoptotic signals through both mitochondria-dependent and independent mechanisms [5]. Additionally, its functional receptors, death receptor 4 (DR4) and 5 (DR5), are found in a broader spectrum of tissue than other TNFSF receptors [6].

TRAIL can bind two functional receptors (DR4 and DR5) and three decoy receptors (DcR1, DcR2 and

osteoprotegerin (OPG)). Functional receptors can transmit death signals to the cytoplasm, whereas the decoy receptors are not capable of doing so. Moreover, there is evidence showing that decoy receptors can trigger the formation of some antiapoptotic factors and contribute to apoptotic resistance [7,8]. Additionally, intracellular inhibitor of apoptosis proteins (IAPs), cIAP-1, cIAP-2 and XIAP, and the FLICE-like inhibitory protein (FLIP) play an antiapoptotic role. IAPs function by inhibiting caspase-9 activation through physical binding [9], whereas FLIP competes with pro-caspase-8 [10].

Hypoxia is a deficiency of oxygen in tissues and it has important effects on tumor mass [11]. Exposure to hypoxia leads to tissue injuries, and long-term acute hypoxia can cause death in most animals [12,13]. Hypoxia might act as a natural selection mechanism, which leads to the survival of more resistant mutant cells. Angiogenetic malformations cause hypoxic regions to develop solid tumors due to the three-dimensional structure of the tumor masses. Apoptotic resistance develops in these hypoxic regions. Therefore, these areas can play an important role in tumor progression [11]. It is also known that solid tumors develop resistance to chemotherapy and radiotherapy under hypoxia [14], and studies have shown that hypoxia is a factor that can induce resistance against TRAIL-mediated cell death in cancer cells [15-17]. The effects of hypoxia on TRAIL-mediated cell death mechanisms are still unclear. It seems that hypoxia-resistant animals can be exceptional models for understanding the effects of hypoxia on TRAIL-mediated cell death.

Blind mole rats (BMRs) are rodents belonging to the Spalacidae family that spend their lives in hypoxic underground galleries [18,19]. They have an extremely long lifespan of over 20 years, and spontaneous tumor developments have never been reported throughout forty years of experimental examination [20]. Many studies propose that BMRs have gained anticancer features over millions of years of evolution [21]. BMRs have a unique idiosyncratic p53 form (sp53) that has evolved on the 174th amino acid position, which is often mutated in terminal human cancers. It is known that this modification amplifies the promotor activation of Apaf-1 up to three-fold [22,23]. This sp53 form also leads to autophagy, as well as caspase-dependent cell death [24]. BMRs also have a characteristic gene form of nuclear respiratory factor 1 (Nrf1) [19]. This characteristic form is related to high antioxidant enzyme activity and contributes to exceptional protection against ROS [19]. Interestingly, while cultured fibroblast cells isolated from BMRs do not show contact inhibition, these cultured cells can commit suicide collectively via a typical cell death known as concerted cell death [23]. Furthermore, due to their phylogenetic position, BMRs are a suitable model organisms [21].

In the present study, we first aimed to investigate the mRNA expression levels of TRAIL-mediated cell death related genes (cIAP-1, cIAP-2, XIAP, DcR1, DcR2, OPG, FLIP and TRAIL) under hypoxia, and to determine the sequences of cIAP-1, cIAP-2, XIAP, OPG, FLIP, and TRAIL in *Nannospalax nehringi*.

MATERIALS AND METHODS

Housing of BMRs

This study was carried out with 9 male subadult (11-15 months) *Nannospalax nehringi*, captured in Kars city, Turkey. The BMRs were housed in individual cages at the Kafkas University, Veterinary Medicine Faculty, Department of Physiology Research Laboratory, at 21-23°C, 70-75% humidity and in constant darkness. The animals were fed with fresh vegetables, fruit and seeds *ad libitum*. For the laboratory adaptation, the animals were housed under the same conditions for three months. Research ethics committee approval was provided for the capture of the *Nannospalax nehringi*, permission No. 72784983-488.04-23381, from the Republic of Turkey Ministry of Forestry and Water Affairs, Directorate of Nature Conservation and National Parks in regard to 2015/129, numbered in conjunction with KAU-HADYEK (Kafkas University Local Ethics Committee for Animal Experiments). All experimental procedures were conducted in accordance with the European Council Directives (86/609/EEC).

Experimental design

After three months, BMRs were randomly divided into two groups, normoxic and hypoxic. BMRs in the normoxic group (n=4) were housed under normoxic conditions, and the hypoxic group (n=5) was housed in a hypoxic glovebox chamber, maintained with a 7% O_2 + 93% N_2 gas mixture (20 L/min) for 52 h. During the

52-h experimental period, no reflex loss nor behavioral changes were observed in the BMRs. The animals in both groups were kept under constant dark conditions (24D:0L) and killed by cervical dislocation under sevoflurane anesthesia. Liver and brain tissues were homogenized in 1 mL TRI reagent (Sigma-Aldrich, USA) also known as TRIzol, a monophasic solution of phenol and guanidinium isothiocyanate, and immediately stored at -80°C.

cDNA (complementary DNA) library

Total RNA was isolated by the TRIzol method, using TRI Reagent. The quality and quantity of the RNA samples were determined with a nanodrop spectrophotometer (Avans Biotechnology, Taiwan) at a 260/280 nm ratio. For the quality assay, 10 µL of total RNA were separated by electrophoresis in a 1% agarose gel. A RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to obtain the cDNA library from the total RNA according to the manufacturer's instructions.

RT-PCR (reverse transcription polymerase chain reaction) and sequencing

No sequence information was found in the GenBank database for the described genes of *N. nehringi* or the Spalacidae family. Reliable gene sequences from the family Rodentia on the NCBI were used to design the primer sets. Amplification target genes were carried out using the following primers: cIAP-1, forward (5'-ATGGACAAAACTGTCTCCCAGAGACTC-3') and reverse (5'-TCATGAGAGAAATGTGCGCACAGTCCC-3'); cIAP-2 forward (5'-ATGACATGGTTCAAGACAGCGCCTTTCTA-3') and reverse (5'-TCAGGAGAGAAATGTGCGCACTGTGCCC-3'), and; XIAP, forward (5'-ATGACTTTTAACAGTTTTGAAGGATCTAGAA-3') and reverse (5'-TTAAGACATAAAAATTTTTGCTTGAACGTAA-3'); DcR1, forward (5'-ATGGCCCGGATCCCCAAGACCCTAAAGTTCGTC-3') and reverse (5'-TCAAACAAACACAATCAGAAGCACAATTAGAAC-3'); DcR2, forward (5'-ATGGGACTTTGGGGACAAAGCGTCCCGACCGC-3') and reverse (5'-TCACAGGCAGGACGTAGCAGAGCCTGCCTCAT-3'); OPG, forward (5'-ATGAACAAGTGGCTGTGCTGCGCACTCCTGG-3') and reverse (5'-TTATAAGCAGCTTATTTTCACGGATTGAACC-3'); FLIP, forward (5'-ATGGCCCTGAGCACTGTGTCTGCTGAGG-3') and reverse (5'-TCATGTGTGAGACAGGATGAGTTTCTTCC-3'); TRAIL, forward (5'-ATGCCTTCCTCAGGGGCCCTGAAGGACCTC-3') and reverse (5'-TTAGTTAATTAAAAAGGCTCCAAAGAAGCT-3'). A positive control (β-actin) was included in each reaction for normalization [25]. The PCR conditions were pre-denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The final extension step was carried out at 72°C for 5 min. All samples were run on a 1% agarose gel with a 100-bp DNA molecular weight marker, and amplicons were visualized under UV. Sequence analysis of the obtained bands was performed by the Sanger method.

Statistical and informatics analyses

For statistical analyses, band images were converted into numeric data using ImageJ 1.51j8 software. The Shapiro-Wilk normality test was applied to all data. To determine the statistical differences, a Mann-Whitney U test was applied to nonparametric values and an Independent Samples t-Test was applied to parametric values. All statistical analyses were performed with IBM SPSS Statistics 20.0.0 software, and $P<0.05$ was considered as significant.

The obtained gene sequences were analyzed, edited and aligned using Geneious R 9.1.8 Software [26]. Trimmed and edited bases were translated to an amino acid sequence with standard genetic code and translation frame parameters. MUSCLE Alignment and BLAST tools in Geneious R 9.1.8 were used to compare nucleotide and amino acid sequences. The Tandem Repeats Finder web server was used to determine the repeated motifs [27]. Additional sequences from the GenBank nucleotide database were obtained and used, and gene regions and domains were associated with gene regions of reference sequences that have close sequence similarity with *N. nehringi*. The obtained partial sequences were checked and submitted to GenBank with the following accession numbers: MN610568 (cIAP-1), MN610569 (cIAP-2), MN610573 (XIAP), MN610570 (FLIP), MN610571 (OPG), and MN610572 (TRAIL). DcR1 and DcR2 sequences were not submitted to GenBank, since no reference sequences were found for these genes.

RESULTS

Comparison of mRNA expression levels

Messenger RNA expression levels of the DcR1, DcR2, cIAP-1, cIAP-2, XIAP, FLIP, OPG and TRAIL genes in the brain and lung tissues of *N. nehringi* under hypoxia and normoxia are displayed in Fig. 1. (A: parametric tests; B: nonparametric tests), and electrophoresis band images are shown in Fig. 2. Our results indicate that relative mRNA expression levels of cIAP-2, OPG and FLIP increased in lung tissue and OPG, FLIP, DcR2, cIAP-1, cIAP-2, XIAP, DcR1 and TRAIL increased in brain tissue, while mRNA expression levels of DcR1, DcR2 and TRAIL decreased in lung tissue under hypoxia. A statistically significant difference was only determined in the lung cIAP-2 mRNA level (P<0.05) (Fig. 1A).

Gene sequence analysis

The 660-bp-long partial coding sequence region (CDS) of the cIAP-1 gene in *N. nehringi* was aligned by translation into an amino acid sequence (Fig. 3). The sequence was most similar (65.60%) to *Mus musculus* (NP_031491). Our results indicated that the cIAP-1 gene of *N. nehringi* had 10 characteristic amino acid mutations (L, A, L, K, N, K, L, T, D and R, respectively) and 1 isoleucine (I) insertion (Fig. 3) on locations of the BIR domains that are protected in the other species. Additionally, 11 mutations (A, R, P, S, S, S, F, Q, H, F and N), 3 deletions and 6 insertions (L, S, W, V, A, and S, respectively) of amino acids were found in the part between the baculovirus IAP repeat (BIR) domains of the cIAP-1 gene. Moreover, a peptide-binding groove amino acid located on the BIR domain was mutated from methionine (M) to leucine (L). Amino acid insertions were also detected in the alignment of the partial CoDing Sequences (CDS) of cIAP-2 (543 bp) and XIAP (384 bp) in *N. nehringi* were as follows: 7 amino acid insertions in cIAP-2 (K, P, G, K, I, P and F, respectively) (Fig. 4) and 3 amino acid insertions in XIAP (Fig. 5) (H, Q and Q, respectively). Moreover, some of these amino acid insertions were found on the BIR domains of the cIAP-2 (4 insertions) and XIAP (4 insertions) genes. Additionally, the partial cIAP-2 gene sequence of *N. nehringi* was found to be more similar (96.13%) to *Mus musculus* (NP_031490), while XIAP was more similar (96.09%) to *Rattus norvegicus* (NP_071567). In the confirmation analyses, no repeated motifs were found.

Multiple alignments of the partial CDS sequence translation of the OPG (528 bp) gene in *N. nehringi* are shown in Fig. 6. Four characteristic amino acid mutations (G, G, P and R, respectively) were detected on the OPG gene, and the closest similarity (96.60%) was

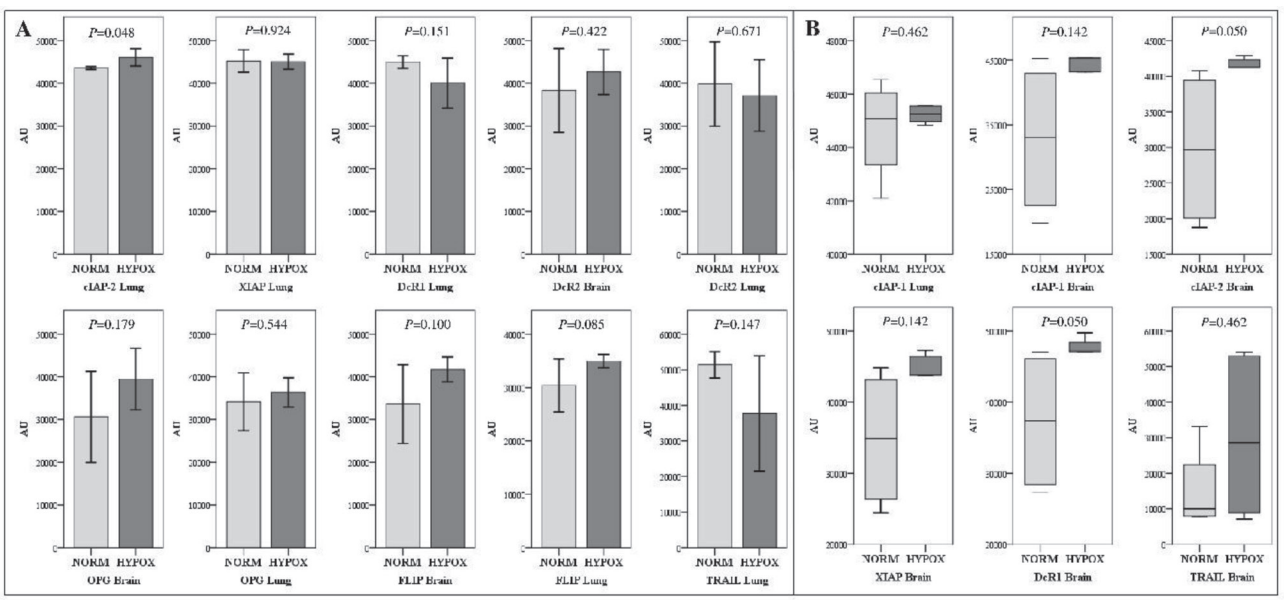

Fig. 1. Effects of hypoxia on mRNA expression levels of cIAP-1, cIAP-2, XIAP, DcR1, DcR2, FLIP, OPG and TRAIL genes in brain and lung tissues of *Nannospalax nehringi*. **A** – Results of parametric tests (Independent Samples t-Test), the bar graphs were created using mean±SD; **B** – Results of nonparametric tests (Mann-Whitney U Test), the box plot graphs were created using median, maximum, minimum, first quartile and third quartile (AU – arbitrary units).

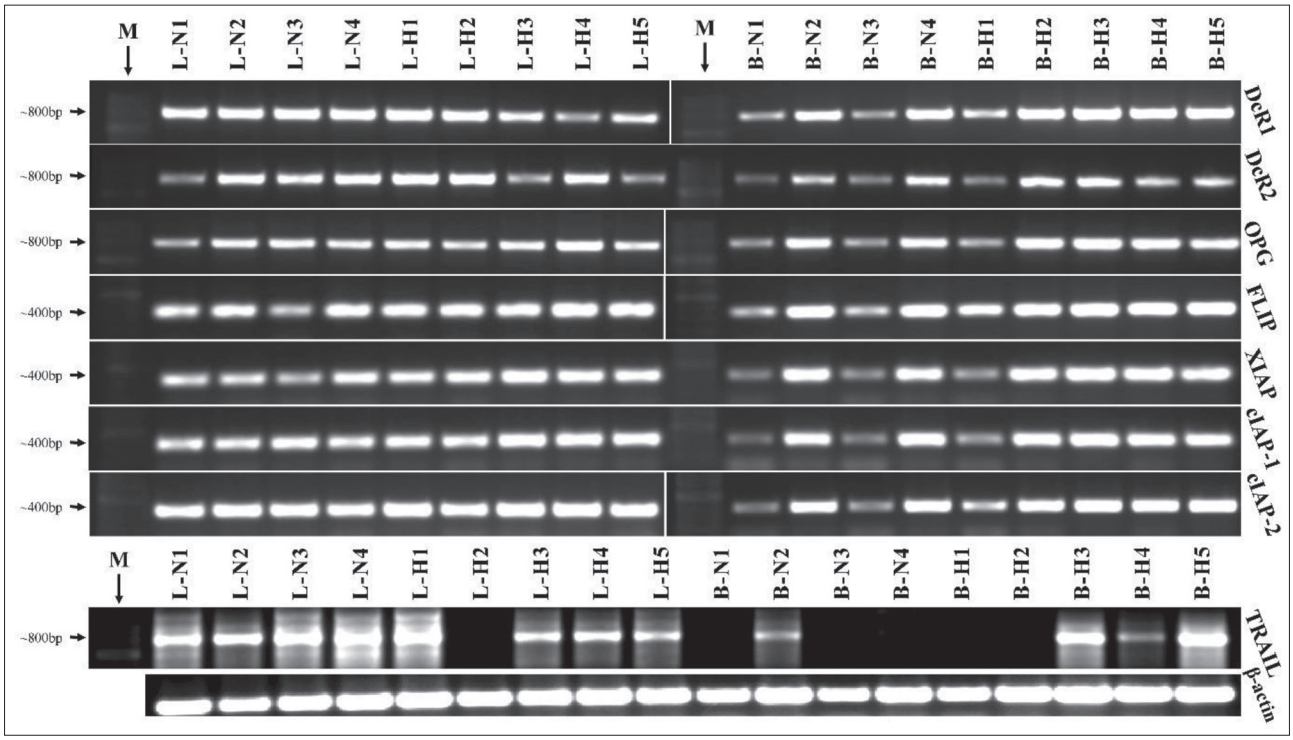

Fig. 2. Agarose gel electrophoresis images of cIAP-1, cIAP-2, XIAP, DcR1, DcR2, FLIP, OPG and TRAIL genes under UV. All samples were run on 1% agarose gel for 60 min at 100 V with a 100-bp DNA molecular weight marker. (L – lung, B – brain, M – 100 bp marker).

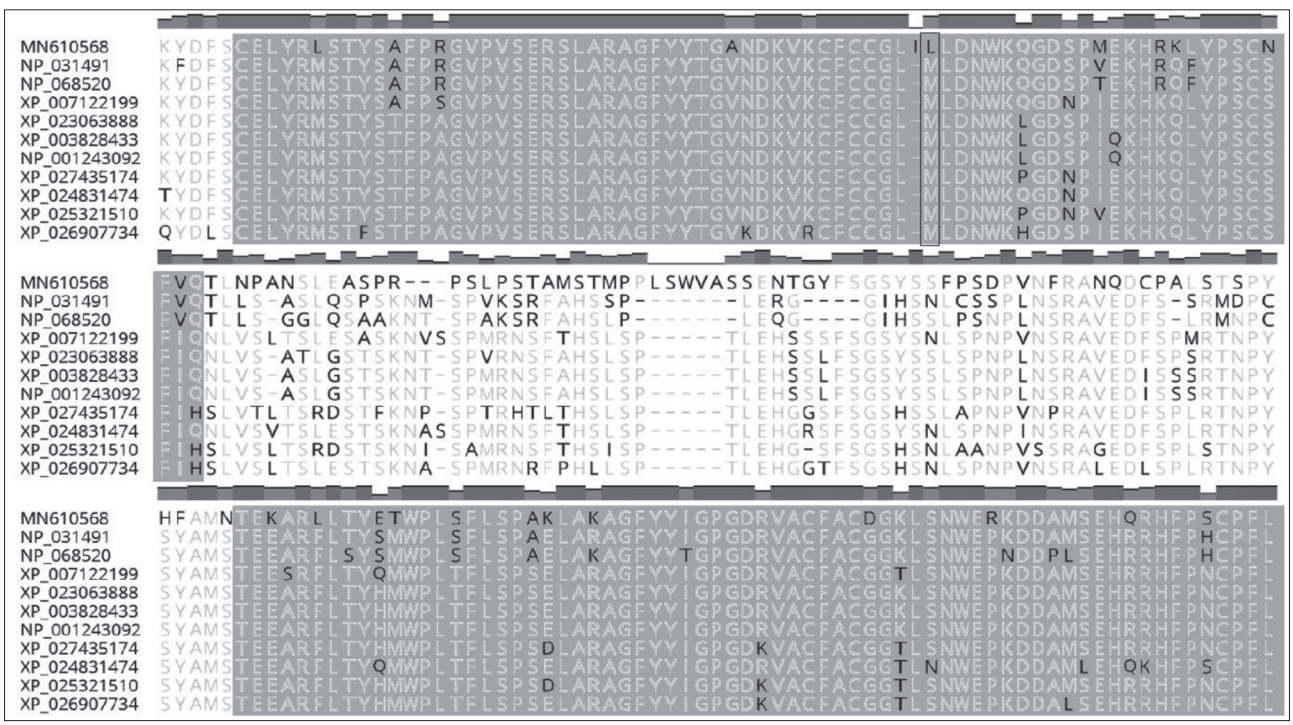

Fig. 3. Multiple alignments (MUSCLE) of the amino acid translation sequence of the cIAP-1 gene of *Nannospalax nehringi*. Grey regions demonstrate BIR domains, and the box shows the peptide binding groove amino acid of the gene (NP_031491: *Mus musculus*, NP_068520: *Rattus norvegicus*, XP_007122199: *Physeter catodon*, XP_023063888: *Piliocolobus tephrosceles*, XP_003828433: *Pan paniscus*, NP_001243092: *Homo sapiens*, XP_027435174: *Zalophus californianus*, XP_024831474: *Bos taurus*, XP_025321510: *Canis lupus dingo*, XP_026907734: *Acinonyx jubatus*).

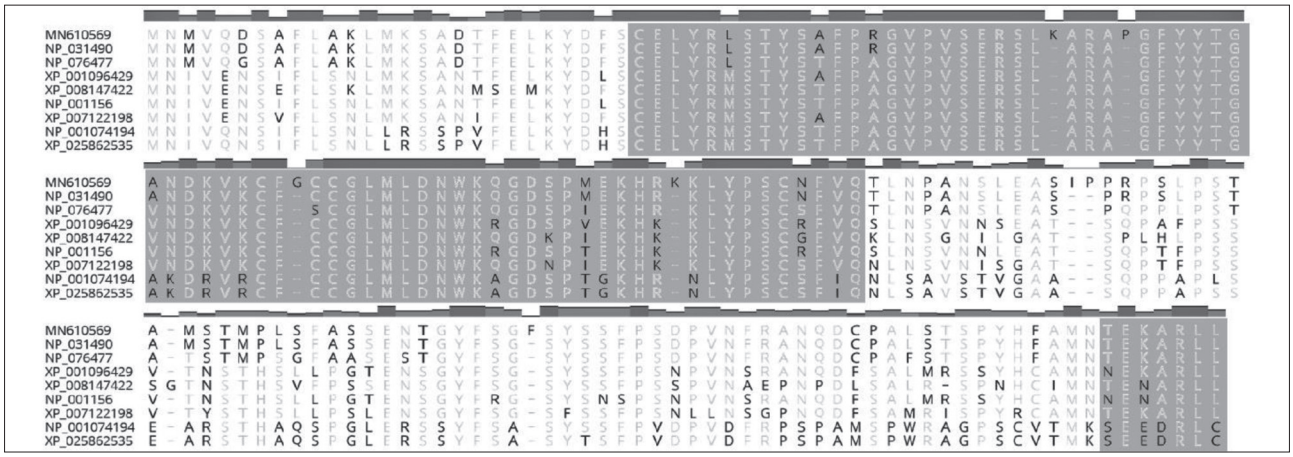

Fig. 4. Multiple alignments (MUSCLE) of the amino acid translation sequence of the cIAP-2 gene of *Nannospalax nehringi*. Grey regions demonstrate BIR domains of the gene (NP_031490: *Mus musculus*, NP_076477: *Rattus norvegicus*, XP_001096429: *Macaca mulatta*, XP_008147422: *Eptesicus fuscus*, NP_001156: *Homo sapiens*, XP_007122198: *Physeter catodon*, NP_001074194: *Canis lupus familiaris*, XP_025862535: *Vulpes vulpes*).

Fig. 5. Multiple alignments (MUSCLE) of the amino acid translation sequence of the XIAP gene of *Nannospalax nehringi*. Grey regions demonstrate BIR domains of the gene (NP_071567: *Rattus norvegicus*, XP_021008861: *Mus caroli*, NP_033818: *Mus musculus*, XP_016799263: *Pan troglodytes*, NP_001158: *Homo sapiens*, XP_030662718: *Nomascus leucogenys*, XP_021096230: *Heterocephalus glaber*).

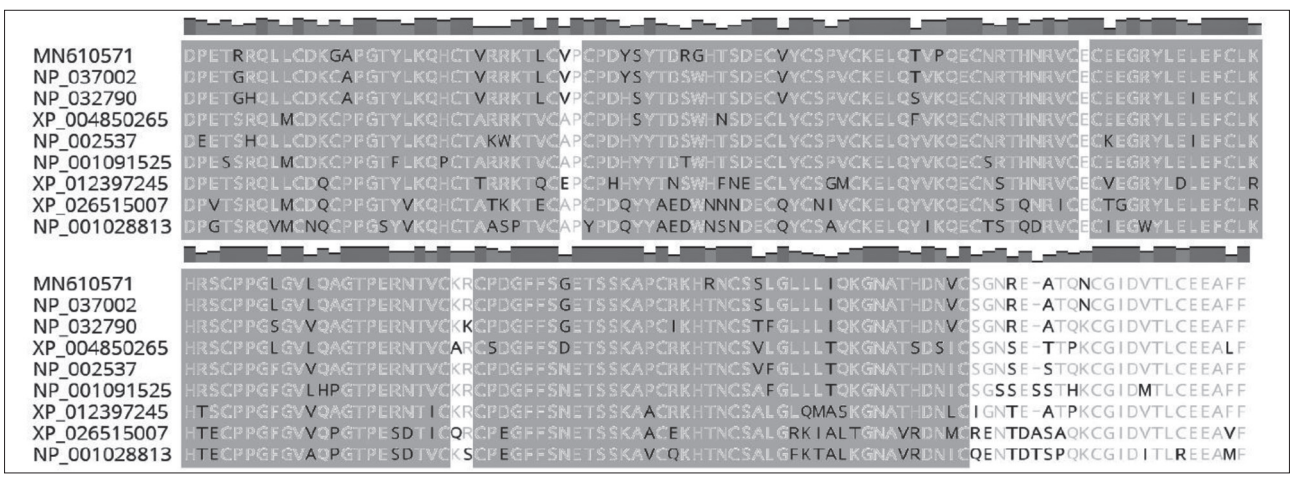

Fig. 6. Multiple alignments (MUSCLE) of the amino acid translation sequence of the OPG gene of *Nannospalax nehringi*. Grey regions show cysteine-rich regions of the gene (NP_037002: *Rattus norvegicus*, NP_032790: *Mus musculus*, XP_004850265: *Heterocephalus glaber*, NP_002537: *Homo sapiens*, NP_001091525: *Bos taurus*, XP_012397245: *Sarcophilus harrisii*, XP_026515007: *Terrapene carolina triunguis*, NP_001028813: *Gallus gallus*).

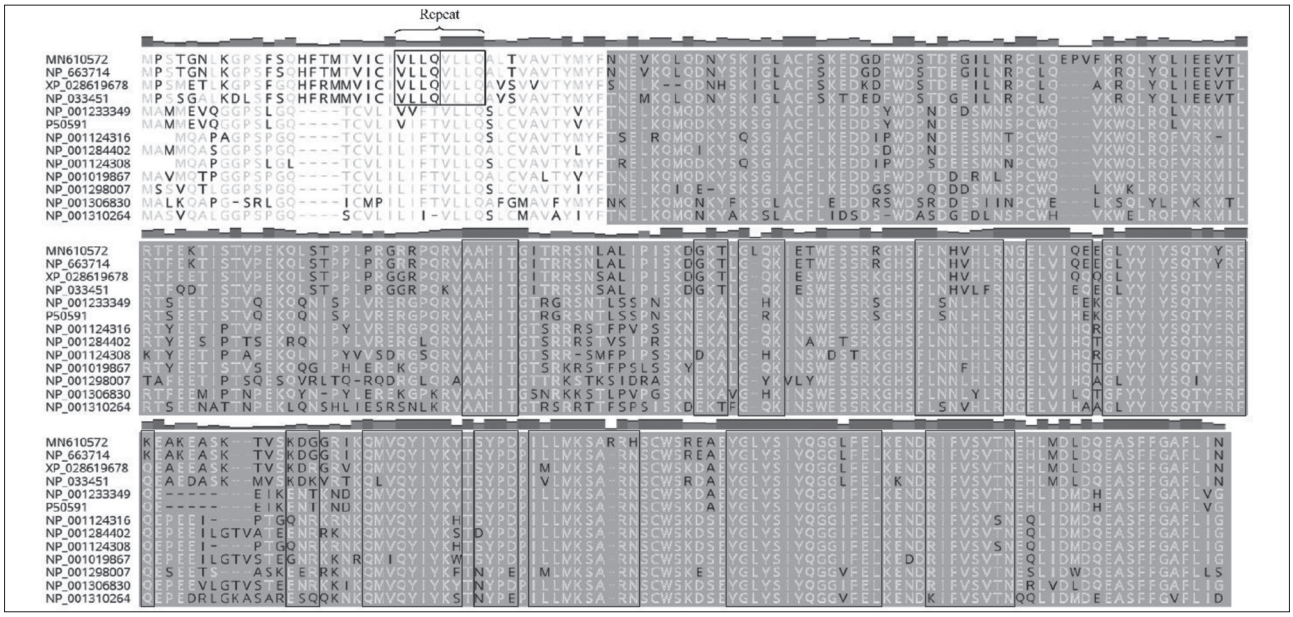

Fig. 7. Multiple alignments (MUSCLE) of the amino acid translation sequence of the TRAIL gene of *Nannospalax nehringi*. Grey regions demonstrate the topological domain of the gene, and the beta-strand regions are shown with boxes. Conserved repeat amino acids are also noted (NP_663714: *Rattus norvegicus*, XP_028619678: *Grammomys surdaster*, NP_033451: *Mus musculus*, NP_001233349: *Pan troglodytes*, P50591: *Homo sapiens*, NP_001124316: *Felis catus*, NP_001284402: *Equus caballus*, NP_001124308: *Canis lupus familiaris*, NP_001019867: *Sus scrofa*, NP_001298007: *Cavia porcellus*, NP_001306830: *Bos taurus*, NP_001310264: *Erinaceus europaeus*).

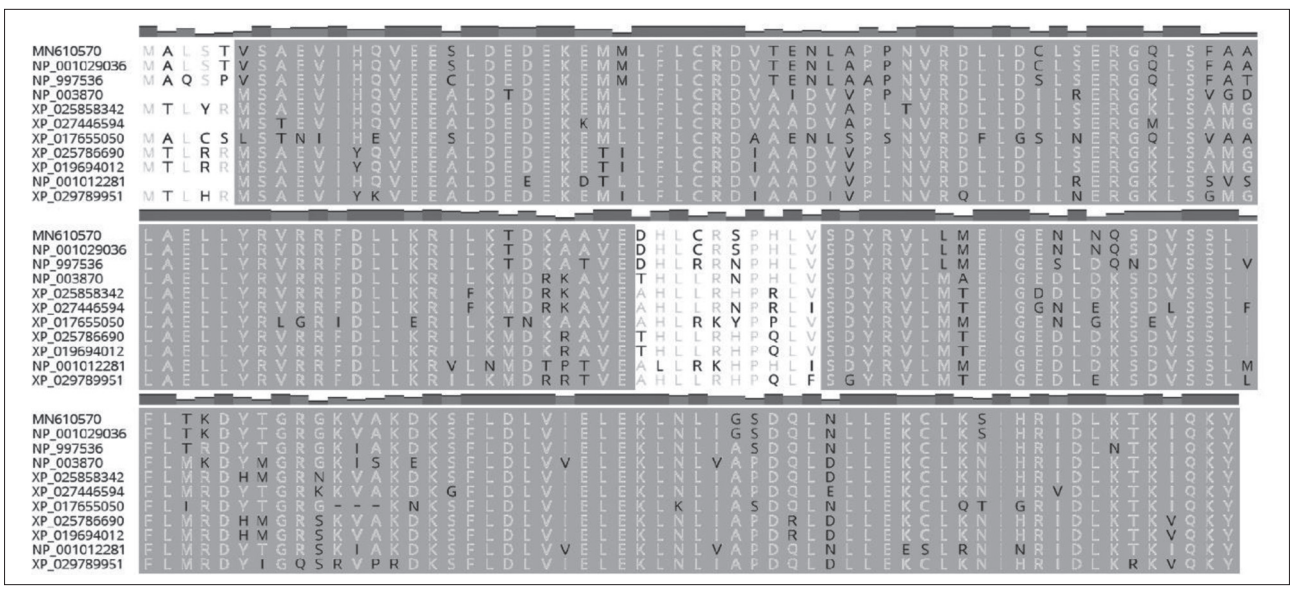

Fig. 8. Multiple alignments (MUSCLE) of the amino acid translation sequence of the FLIP gene of *Nannospalax nehringi*. Grey regions show the death effector domains of the gene (NP_001029036: *Rattus norvegicus*, NP_997536: *Mus musculus*, NP_003870: *Homo sapiens*, XP_025858342: *Vulpes*, XP_027446594: *Zalophus californianus*, XP_017655050: *Nannospalax galili*, XP_025786690: *Puma concolor*, XP_019694012: *Felis catus*, NP_001012281: *Bos taurus*, XP_029789951: *Suricata suricatta*).

detected with *R. norvegicus* (NP_037002). Moreover, all mutations were located in the TNFR cysteine-rich regions, and the mutation points were protected in all the sequenced species (Fig. 6). No repeat motifs were detected in the sequenced region of the OPG gene.

The complete CDS region of the TRAIL gene (891 bp) was sequenced in *N. nehringi* and translated into an amino acid sequence. Multiple alignments of the TRAIL amino acid sequence are shown in Fig. 7. Our results indicate that the TRAIL gene in *N. nehringi*

shares close similarity (97.63%) with *R. norvegicus* (NP_663714). The sequence had 5 insertions (E, P, V, L and R, respectively) and 1 mutation (H) of amino acids compared with the other species. However, 3 amino acid insertions (E, P and V, respectively) were sequential and located on the topological domain, and the other 2 insertions occurred on the beta-strand regions. The histidine (H) amino acid located on the beta-strand region was conserved in all species as asparagine (N) (Fig. 7). In addition, the CDS region of the TRAIL gene of *N. nehringi* was found to contain a repeat motif, repeated 2.1 times and 12 bp in length (Fig. 7). This motif was also observed in the other three sequences (NP_663714, XP_028619678 and NP_033451).

The amino acid sequence and multiple alignment results of the translated partial FLIP gene (528 bp) in *N. nehringi* are shown in Fig. 8. No characteristic amino acid mutation, deletion, insertion or repeated motif was detected, and the presented sequence also had constant harmony (100%) with *R. norvegicus* (NP_001029036).

DISCUSSION

As a programmed cell death type, TRAIL-mediated cell death is capable of killing cancer cells selectively in both mitochondria-dependent and independent manners [5]. Recent studies have focused on clinical trials of a useful initiator agent, ONC201, for TRAIL-mediated apoptosis, as it is not cytotoxic for healthy cells and is also p53-independent [28-31]. Some reports have indicated that hypoxia has negative effects on TRAIL-mediated cell death [14,17]; therefore, hypoxia is considered an important factor in causing resistance to apoptotic cell death, including TRAIL-mediated apoptosis [32–34]. Exposure to long-term or severe hypoxia causes different types of damage and even death in most animals [12,13]. However, hypoxia is a natural selection mechanism for tumor cells and provokes the cells to be resistant to apoptosis [35]. Moreover, it is known that hypoxia can serve to develop chemotherapy- and radiotherapy-resistant mutant cells in hypoxic regions of the tumor mass [11,14].

BMRs are hypoxia-tolerant and cancer-resistant rodents that have unique genetic anticancer mimics [19,22-24]. However, the TRAIL-mediated cell death properties in BMRs remain unknown. In the present study, we compared the mRNA expression profiles of cIAP-1, cIAP-2, XIAP, DcR1, DcR2, FLIP, OPG and TRAIL genes under normoxia and hypoxia, and examined the sequences of cIAP-1, cIAP-2, XIAP, FLIP, OPG and TRAIL genes in *N. nehringi*.

Caspases are zymogen enzymes that mediate the process of programmed cell death, and the undesirable activation of pro-caspases results in unwanted cell death. Therefore, keeping the activation of the caspase cascade under control is of vital importance for organisms. IAPs protect cells against massive apoptotic cell death by inhibiting caspases, and their conserved homologs are found in many taxa, such as fungi, vertebrates and invertebrates [36,37]. The upregulation of IAP proteins is associated with cell survival; thus, IAP members are considered as a resistance marker of cancer treatment and, moreover, apoptotic resistance can break down via downregulation of IAPs [38,39]. Hypoxia is also an important regulator for IAPs. Cells upregulate IAP genes under hypoxia to avoid apoptosis-mediated tissue injuries. It was shown that the upregulation of cIAP-1 [40], cIAP-2 [41-43] and XIAP [44] has a protective role, avoiding stress-derived damage from hypoxia, and that there is also an inverse correlation between XIAP and caspase-3 in ischemic rat brain [45]. In *N. nehringi*, IAPs are upregulated under hypoxia; moreover, our results also indicate that the brain is under tighter control than the lung. It has also been determined that rat fibroblasts hardly upregulate the expression of cIAP-2 under hypoxia, and our results are in agreement with this data. IAPs also have different binding affinities for target proteins via BIR domains [40], which are responsible for their anti-caspase activity [44], with this ability having an important role in the action of therapeutics of IAPs [46]. We also detected several characteristic mutations and insertions of amino acids on the BIR domains of the cIAP-1, cIAP-2 and XIAP genes in *N. nehringi*; however, we could not determine the functions of these characteristic mutations and insertions in the anti-caspase activity of IAPs in *N. nehringi*.

FLIP is another negative regulator of apoptosis. As a dominant-negative inhibitor of caspase-8, FLIP competes with pro-caspase-8 and inhibits the cascade of caspases in the cytoplasm. Therefore, overexpression of FLIP protects cells from cell death. Cancer cells escape TRAIL-mediated cell death via FLIP upregulation, and FLIP overexpression is related to chemotherapeutic

resistance [10]. Additionally, hypoxia is a key factor that induces cellular FLIP expression, thus contributing to cell survival [47]. We observed that hypoxia has a similar effect on FLIP gene expression in *N. nehringi* and no specific characteristic amino acid insertions or deletions were detected on the gene. These results imply that FLIP can be a protected gene among species, but its complete sequence in *N. nehringi* must be elucidated before drawing more accurate conclusions.

DcR1 and DcR2 are ligand (TRAIL)-binding decoy receptors, but they have no ability to transmit death signals into the cytoplasm because they lack a functional death domain [48]. OPG is also considered as another decoy receptor for TRAIL, but it has a soluble form [48]. The prime function of the decoy receptors is to reduce the amount of ligand reaching the cell surface by competing with functional receptors. Thus, it has been hypothesized that upregulation of decoy receptors is one of the factors that causes resistance to TRAIL-mediated cell death [38,49]. Hypoxia has the ability to upregulate the expression of DcR1 [50], DcR2 [33,50] and OPG [51]. In *N. nehringi*, we observed that hypoxia moderately induces DcR1, DcR2 and OPG upregulation in the brain and OPG upregulation in the lung, while decreasing DcR1 and DcR2 in lung tissue. This corresponds to previous results demonstrating that the upregulations of DcR1 and DcR2 have a protective role on neural cells under hypoxia [52]. Our results match these results and suggest that decoy receptors also have a protective role against hypoxia-mediated injuries in *N. nehringi* brain. We also detected characteristic mutations on the partial sequence of the *N. nehringi* OPG receptor, located on cysteine-rich domains of the gene. Cysteine-rich domains are conserved units found in TNFSF receptors with a characteristic motif, and have a function in protein-protein interaction, as these motifs also include ligand-binding sites [53,54]. The possible functions of amino acid mutations on the conserved positions in the OPG gene of *N. nehringi* are not known, but characteristic features of amino acids affect the solubility of proteins [55,56].

TRAIL is the most operable member of the TNFSF family because it is p53-independent, and triggers the apoptotic cascade via both mitochondria-dependent and independent mechanisms. Most tumors have hypoxic compartments, and these units produce chemotherapy- and radiotherapy-resistant mutant cells. Furthermore, hypoxia is a regulatory factor for apoptotic cell death, including TRAIL-mediated cell death. Although hypoxia is known to have inhibitory effects on TRAIL-mediated cell death [17,57], results also suggest that it is inductive in cancer cells [58]. These different results could be associated with different mutational aspects of the cancer cells studied. On the other hand, it is known that TRAIL expression is enhanced in response to hypoxia-ischemia in the brain [50,51]. Similarly, we detected a relative increase in mRNA expression level of the TRAIL gene in *N. nehringi* brain tissues under hypoxia, but the gene expression had a decreasing trend in lung tissues. In terms of protein characteristics of the TRAIL gene in *N. nehringi*, we detected many mutations and sequential or single insertions, some of which were located on the β-strand regions of the topological domain. β-strand regions are secondary structure elements of proteins [59], and mutations on these regions affect the structure of mature proteins; therefore, the solubility and affinity of the proteins are changed [55,60]. However, we still do not know what advantages these differences afford in terms of the solubility, affinity and efficacy of the ligand.

CONCLUSIONS

The genes related with TRAIL-mediated cell death in *N. nehringi* brain are regulated by hypoxia, and this regulation also seems to protect the brain tissue of *N nehringi* from the possible effects of hypoxia, as in other species. Our findings also indicate that hypoxia regulates TRAIL-mediated cell death-related proteins in a different way in *N. nehringi* lung tissues; however, our parameters and results are insufficient to comment on the advantages or disadvantages of this. We also detected many mutations and insertions of amino acids in the genes, but we have no information about the functions of these variations. Therefore, further recombinant studies should be conducted to understand the functions of these variations.

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