Published in final edited form as:

Histochem Cell Biol. 2018 July ; 150(1): 77–82. doi:10.1007/s00418-018-1669-6.

Detection of pro-apoptotic Bax 2 proteins in the human cerebellum

Adriana Mañas¹, Aislinn Davis¹, Sydney Lamerand¹, Jialing Xiang¹

¹Department of Biology, Illinois Institute of Technology, 3101 South Dearborn Street, Chicago, IL 60616, USA

Abstract

Bax 2 is a pro-apoptotic protein originally discovered in colon cancer patients with high microsatellite instability. Unlike most pro-apoptotic Bax family members, Bax 2 mediates cell death through a non-mitochondrial caspase 8-dependent pathway. In the scope of analyzing the distribution of Bax 2 expression in human tissues, we examined a panel of human brain samples. Here, we report four cerebellar cases in which the subjects had no neurological disorder or disease documented. We found Bax 2 positive cells scattered in all areas of the cerebellum, but most strikingly concentrated in Purkinje cell bodies and dendrites. Two out the four subjects tested had strong Bax 2-positive staining in nearly all Purkinje cells; one was mainly negative; and one had various levels of positive staining within the same sample. Further genetic analysis of the Purkinje cell layer, collected by microdissection from two subjects, showed that the samples contained G7 and G9 *Bax* microsatellite mutations. Both subjects were young and had no diseases reported at the time of death. As the distribution of Bax 2 is consistent with that known for Bax α , but in a less ubiquitous manner, these results may imply a potential function of Bax 2 in Purkinje cells.

Keywords

Bax 2; Cerebellum; Purkinje cells; Apoptosis; Human brain

Introduction

The cerebellum is the area of the brain responsible for the modification of motor commands and is involved in balance, posture, voluntary movements, motor learning, and more (Koziol et al. 2014; Bernard and Seidler 2014). The cerebellar cortex is composed of three layers: the granular layer, the Purkinje cell layer, and the molecular layer. The dendritic trees from Purkinje cells extend into the molecular layer (Casoni et al. 2017). Recent studies showed that Purkinje cells play a role in information storage for learned response sequences in coordination of motor behaviors (Jirenhed et al. 2017). It also has been shown that Purkinje

Jialing Xiang, xiang@iit.edu.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00418-018-1669-6>) contains supplementary material, which is available to authorized users.

Conflict of interest The authors declare that they have no conflict of interest.

cells may be involved in the pathophysiology of schizophrenia (Picard et al. 2007; Mothersill et al. 2016).

Bax is a pro-apoptotic Bcl-2 family member that is ubiquitously distributed throughout all human tissues (Krajewski et al. 1994; Penault-Llorca et al. 1998). Bax plays a critical role in development and in tissue homeostasis, and its dys-regulation can lead to many diseases. Due to its involvement in the development and progression of neurodegenerative diseases and ischemic insults, expression and distribution of Bax in the brain have been widely studied (Hara et al. 1996; Fan et al. 2001; Vogel 2002; Dorszewska et al. 2004; Jung et al. 2008; Didonna et al. 2012; Garcia et al. 2013). Bax is known to have several functional isoforms, but only the distribution of the canonical isoform, Bax α , has been fully studied. Bax α is present in all the different areas of the brain, mainly in neuronal bodies, with very low to no presence in glial cells (Hara et al. 1996; Vogel 2002; Didonna et al. 2012). Cerebellar Purkinje cells and hippocampal neurons have the highest levels of Bax α (Hara et al. 1996; Vogel 2002; Casoni et al. 2017), which is believed to be the reason why these cells are so vulnerable to ischemic insults (Krajewski et al. 1995). Apart from its apoptotic role, other physiological functions of Bax α in the brain are unknown.

Bax 2 is a unique isoform of the Bax subfamily originally discovered in colorectal cancer patients with high microsatellite instability (MSI-H) (Haferkamp et al. 2012; Zhang et al. 2015). It is generally believed that generation of Bax 2 requires a microsatellite frameshift mutation in combination with an alternative splicing event that restores the reading frame. Like Bax α , Bax 2 is pro-apoptotic and has similar characteristics, such as binding with Bcl-2; however, Bax 2 does not target mitochondria, and instead activates a caspase 8-dependent death pathway (Haferkamp et al. 2012; Zhang et al. 2014; Mañas et al. 2017). In the process of analyzing the expression and distribution of Bax 2 throughout the human body, we examined several tissue sections of human brain. Here, we report the expression and distribution of Bax 2 in the cerebellum of four young and healthy human subjects.

Materials and methods

Materials

All tissue sections and tissue microarray slides were commercially obtained from Biomax. All samples were collected less than 6 h postmortem, fixed, and embedded according to standard procedure. All samples were de-identified and assigned with codes. Antibody against Bax 2 was previously generated and validated (Haferkamp et al. 2012; Zhang et al. 2014; Mañas et al. 2018). Further antibody validation for immunohistochemical staining of formalin-fixed paraffin-embedded tissues was performed using Bax 2 antigen peptide competition (Fig. S1).

Immunohistochemistry and tissue analysis

Tissue slides were de-waxed using xylene and rehydrated using graded ethanol solutions. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Slides were incubated in sodium citrate buffer (0.01 M, pH 6.0) at 95 °C for epitope retrieval. After blocking with 5% BSA, CoverWell incubation humidity chambers were used to incubate the

slides with anti-Bax 2 antibody (1:100 in blocking buffer) at 4 °C overnight and then with Biotin-conjugated goat anti-mouse secondary antibody (1:200 in PBS) at room temperature for 2 h. Vectrastain ABC Kit (Vector Laboratories) and ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories) were used for visualization, and Hematoxylin QS (Vector Laboratories) was used for nuclear staining. Finally, slides were dehydrated and fixed using xylene-based mounting media Poly-Mount (Polysciences Inc.). Fluorescence staining was performed as described for the DAB staining until the step of the primary antibody incubation. Slides were then incubated with Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) (1:200) at room temperature for 2 h. Slides were fixed using ProLong Gold antifade reagent (Invitrogen). Slides were scanned using a CRi Panoramic Scan Whole Slide Scanner with a 40x NA 0.95 LWD Zeiss objective on high resolution (down to 0.12 $\mu\text{m}/\text{pixel}$) at the Integrated Light Microscopy Core Facility at the University of Chicago and visualized using Panoramic Viewer 1.15.2. Each slide was analyzed by three independent viewers and samples with debated evaluation were analyzed by a fourth individual. The overall intensity of Bax 2 staining was scored for each sample and divided into four categories: “-” for negative, “+” for weak positive, “++” for intermediate positive, and “+++” for strong staining.

Microdissection and genotyping

The stained tissue sections of cerebellum from subjects 1 and 3 were uncovered using xylene and rehydrated with graded ethanol solutions. The Purkinje cell layer was micro-dissected under a microscope using a sterile needle. The tissue was collected and lysed with Proteinase K. DNA was then isolated using AMPure XP magnetic beads (Beckman Coulter). Sanger sequencing was performed at the DNA Sequencing and Genotyping Facility at the University of Chicago Comprehensive Cancer Center.

Results and discussion

The monomeric form of Bax α is ubiquitously distributed throughout different human tissues, including the brain (Hara et al. 1996). However, distribution of other Bax isoforms is unknown. In the process of screening the tissue distribution of Bax 2, we screened brain tissues from 27 subjects, but cerebellar samples were only available from 4 subjects. We analyzed all four cases and report them here. The patient information about these four subjects is summarized in Fig. 1a. There were two males and two females, all between the ages of 15 and 35 years. All subjects were healthy at the time of death and had no tumors or neuronal diseases reported. All tissues were immunohistochemically stained with an anti-Bax 2 antibody, which has been shown previously to be very specific for Bax 2 and has no cross-staining with parental Bax α (Haferkamp et al. 2012; Zhang et al. 2014; Mañas et al. 2018), and was also validated for IHC staining of formalin-fixed paraffin-embedded tissues (Fig. S1). We detected significant amounts of Bax 2-positive cells in three out of the four subjects. As shown in Fig. 1b, the most significant Bax 2-positive cells were found in the Purkinje cell layer, between the granular and molecular layers. Subjects 3 and 4 had strong Bax 2 staining in almost all Purkinje cells; subject 2, on the other hand, had almost no positive staining observed; and subject 1 had mixed populations of both positive and negative Bax 2 cells in close regions.

Further analysis of the positive samples showed that most Bax 2-positive staining was detected in the Purkinje cell bodies (Fig. 2a), as well as in the dendrites extending into the molecular layer (Fig. 2d–f). In contrast with the strong positive-stained Purkinje cells, we also observed some weaker positive cells scattered throughout the granular layer (Fig. 2b) and the molecular layer (Fig. 2c). The nature of these cells remains to be determined; however, morphologically, they appeared to be granule cells and Golgi cells in the granular layer, and basket cells (large nuclei and close to the Purkinje cell layer) and stellate cells (smaller nuclei and body size) in the molecular layer. Overall, Bax 2 seemed evenly distributed in the cerebellum at low levels, with significantly higher Bax 2-positive staining in the Purkinje cells. These results are consistent with published data for the distribution of Bax α , which also presents higher levels in Purkinje cells. However, the amount and distribution of Bax 2-positive cells, outside of the Purkinje cell layer, was lower and less ubiquitous than for Bax α (Hara et al. 1996).

Bax 2 was previously detected in MSI-H tumors which involve mutations in the *Bax* microsatellite (Haferkamp et al. 2012). As none of these subjects were reported to have any tumors or neurological conditions, we wondered whether they could have a *Bax* microsatellite mutation. Due to limited sample size and variation of sample quality, we were only able to genetically analyze two of the four subjects. The tissue slides were first immunohistochemically stained with anti-Bax 2 antibody, then the Purkinje cell layer was carefully harvested through microdissection under a microscope, as shown in Fig. 3a, and finally genomic DNA was isolated for sequence analysis. A 200 base-pair segment of the *Bax* gene, containing the microsatellite region, was amplified by PCR and subjected to Sanger sequence analysis. As shown in Fig. 3b, in comparison with the wild-type *Bax* microsatellite, which contains a stretch of eight guanines (G8), the sample from subject 1 contains a G9 mutation and the sample from subject 3 contains a G7 mutation. A section of the granular layer of subject 1 was also microdissected. The sequence showed the G9 mutation, as the Purkinje cell layer.

It is generally believed that expression of Bax 2 requires a *Bax* microsatellite G7 mutation and an alternative splicing event, which salvages the frameshift caused by the guanine deletion (Haferkamp et al. 2012). The result from subject 3 seemed consistent with the Bax 2-positive staining, as the G7 mutation was detected. However, a G9 mutation was detected for subject 1, who also had a strong Bax 2 staining in the area dissected, similar to that in subject 3. This cannot be explained by the general rule for Bax 2 expression. Although we have previously shown that the G9 mutation can generate another Bax 2 sub-isoform, Bax 2(G9), which has a similar behavior to Bax 2 (Haferkamp et al. 2013), the antibody used here is specific for Bax 2 and does not cross react with Bax 2(G9). One possibility is that the areas collected by microdissection contained a low number of cells with the G7 mutation, mixed with a significant amount of unstained cells with predominant G9 genotype. Another possibility is that the G8-to-G7 deletion could occur at the transcriptional level by transcriptional slippage, or at the translational level by ribosomal frameshift (Ketteler 2012; Atkins et al. 2016). Both transcriptional slippage and ribosomal frameshift are currently under investigation in our laboratory.

Nevertheless, we report the discovery of pro-apoptotic Bax β proteins in cerebellar neuron cells of young healthy individuals. The similar distribution pattern as canonical Bax α , especially in Purkinje cells, implies a potential physiological function of Bax β in neurons, independently or in compensation for Bax α .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institutes of Health (R15 CA195526).

References

- Atkins JF, Loughran G, Bhatt PR, Firth AE, Baranov PV (2016) Ribosomal frameshifting and transcriptional slippage: from genetic steganography and cryptography to adventitious use. *Nucleic Acids Res* 44: gkw530. 10.1093/nar/gkw530
- Bernard JA, Seidler RD (2014) Moving forward: age effects on the cerebellum underlie cognitive and motor declines. *Neurosci Biobehav Rev* 42:193–207. 10.1016/J.NEUBIOREV.2014.02.011 [PubMed: 24594194]
- Casoni F, Croci L, Cremona O, Hawkes R, Consalez GG (2017) Early Purkinje cell development and the origins of cerebellar patterning In: *Development of the cerebellum from molecular aspects to diseases*. Springer, Cham, pp 67–86
- Didonna A, Sussman J, Benetti F, Legname G (2012) The role of Bax and caspase-3 in doppel-induced apoptosis of cerebellar granule cells. *Prion* 6:309–316. 10.4161/pri.20026 [PubMed: 22561161]
- Dorszewska J, Adamczewska-Goncerzewicz Z, Szczech J (2004) Apoptotic proteins in the course of aging of central nervous system in the rat. *Respir Physiol Neurobiol* 139:145–155. 10.1016/J.RESNP.2003.10.009 [PubMed: 15122998]
- Fan H, Favero M, Vogel MW (2001) Elimination of Bax expression in mice increases cerebellar Purkinje cell numbers but not the number of granule cells. 91:82–91
- Garcia I, Crowther AJ, Gama V, Ryan Miller C, Deshmukh M, Gershon TR (2013) Bax deficiency prolongs cerebellar neurogenesis, accelerates medulloblastoma formation and paradoxically increases both malignancy and differentiation. *Oncogene* 32:2304–2314. 10.1038/ncr.2012.248 [PubMed: 22710714]
- Haferkamp B, Zhang H, Lin Y, Yeap X, Bunce A, Sharpe J, Xiang J (2012) Bax β is a novel bax isoform unique to microsatellite unstable tumors. *J Biol Chem* 287:34722–34729. 10.1074/jbc.M112.374785 [PubMed: 22910913]
- Haferkamp B, Zhang H, Kissinger S, Wang X, Lin Y, Schultz M, Xiang J (2013) Bax β family alternative splicing salvages Bax micro-satellite-frameshift mutations. *Genes Cancer* 4:501–512. 10.1177/1947601913515906 [PubMed: 24386510]
- Hara A, Hirose Y, Wang A, Yoshimi N, Tanaka T, Mori H (1996) Localization of Bax and Bcl-2 proteins, regulators of programmed cell death, in the human central nervous system. *Virchows Arch* 429–429:249–253. 10.1007/BF00198341
- Jirenhd D-A, Rasmussen A, Johansson F, Hesslow G (2017) Learned response sequences in cerebellar Purkinje cells. *Proc Natl Acad Sci USA* 114:6127–6132. 10.1073/pnas.1621132114 [PubMed: 28533379]
- Jung A-R, Kim TW, Rhyu IJ, Kim H, Lee YD, Vinsant S, Oppenheim RW, Sun W (2008) Misplacement of Purkinje cells during postnatal development in Bax knock-out mice: a novel role for programmed cell death in the nervous system? *J Neurosci* 28:2941–2948. 10.1523/JNEUROSCI.3897-07.2008 [PubMed: 18337425]
- Ketteler R (2012) On programmed ribosomal frameshifting: the alternative proteomes. *Front Genet* 3:242 10.3389/fgene.2012.00242 [PubMed: 23181069]

- Koziol LF, Budding D, Andreasen N, D'Arrigo S, Bulgheroni S, Imamizu H, Ito M, Manto M, Marvel C, Parker K, Pezzulo G, Ramnani N, Riva D, Schmähmann J, Vandervert L, Yamazaki T (2014) Consensus paper: the cerebellum's role in movement and cognition. *Cerebellum* 13:151–177. 10.1007/s12311-013-0511-x [PubMed: 23996631]
- Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am J Pathol* 145:1323–1336 [PubMed: 7992838]
- Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. *J Neurosci* 15:6364–6376 [PubMed: 7472401]
- Mañas A, Wang S, Nelson A, Li J, Zhao Y, Zhang H, Davis A, Xie B, Maltsev N, Xiang J (2017) The functional domains for Bax 2 aggregate-mediated caspase 8-dependent cell death. *Exp Cell Res* 359:342–355. 10.1016/J.YEXCR.2017.08.016 [PubMed: 28807790]
- Mañas A, Chen W, Nelson A, Yao Q, Xiang J (2018) Bax 2 sensitizes colorectal cancer cells to proteasome inhibitor-induced cell death. *Biochem Biophys Res Commun* 496:18–24. 10.1016/J.BBRC.2017.12.156 [PubMed: 29291406]
- Mothersill O, Knee-Zaska C, Donohoe G (2016) Emotion and theory of mind in schizophrenia—investigating the role of the cerebellum. *Cerebellum* 15:357–368. 10.1007/s12311-015-0696-2 [PubMed: 26155761]
- Penault-Llorca F, Bouabdallan R, Devilard E, Charton-Bain M-C, Hassoun J, Birg F, Xerri L (1998) Analysis of BAX expression in human tissues using the anti-BAX, 4F11 monoclonal antibody on paraffin sections. *Pathol Res Pract* 194:457–464. 10.1016/S0344-0338(98)80114-3 [PubMed: 9728362]
- Picard H, Amado I, Mouchet-Mages S, Olie J-P, Krebs M-O (2007) The role of the cerebellum in schizophrenia: an update of clinical, cognitive, and functional evidences. *Schizophr Bull* 34:155–172. 10.1093/schbul/sbm049 [PubMed: 17562694]
- Vogel MW (2002) Cell death, Bcl-2, Bax, and the cerebellum. *Cerebellum* 1:277–287. 10.1080/147342202320883588 [PubMed: 12879966]
- Zhang H, Lin Y, Manas A, Zhao Y, Denning MF, Ma L, Xiang J (2014) BaxDelta2 promotes apoptosis through caspase-8 activation in microsatellite-unstable colon cancer. *Mol Cancer Res* 12:1225–1232. 10.1158/1541-7786.MCR-14-0162 [PubMed: 24842234]
- Zhang H, Tassone C, Lin N, Mañas A, Zhao Y, Xiang J (2015) Detection of Bax microsatellite mutations and BaxDelta2 isoform in human buccal cells. *J Cell Sci Ther*. 10.4172/2157-7013.S8-002

Fig. 1.

Bax 2 is expressed at different levels in the cerebella of four different subjects. **a** Table summarizing the information of four subjects. **b** Immunohistochemical staining of tissue sections with anti-Bax 2 antibody. The tissue sections show cerebella from the four subjects in **a**. The non-enlarged tissue samples are presented at the same scale, scale bar 500 μ m. The enlarged tissue areas are presented at the same scale, scale bar 100 μ m. Black arrows point at Purkinje cells presenting various levels of Bax 2 staining

Fig. 2.

Positive Bax 2 cells are observed in all layers of the cerebellum. **a** Immunohistochemical staining of the Purkinje cell layer from subject 1 with an enlarged Bax 2-positive Purkinje cell. **b** Granular layer from subject 3 with Bax 2-positive cells. **c** Molecular layer from subject 3 with several Bax 2-positive cells. **d** Purkinje cell layer and molecular layer from subject 1 with Bax 2-positive Purkinje cells. Black arrows point to Bax 2-positive dendrites. **e, f** Fluorescence immunostaining of cerebellum sections from subject 3 presenting Bax 2-positive staining in Purkinje cell bodies and dendrites. Red arrows point to Bax 2-positive dendrites. Scale bar 50 μ m

Fig. 3.

Genetic analysis of the Bax²-positive Purkinje cell layer from two subjects shows mutations in the *Bax* microsatellite. **a** Immunohistochemical staining of a cerebellum section from subject 1, before and after microdissection of the Purkinje cell layer. Black arrows point to the Purkinje cell layer area dissected. Scale bar 500 μ m. **b** Captions of Sanger sequencing data from the *Bax* microsatellite region of a wild-type (G8) control, subject 1 (G9), and subject 3 (G7)