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Chapter 19

Use of Immunohistochemistry to Determine Expression of Rab5 Subfamily of GTPases in Mature and Developmental Brains

Kwok-Ling Kam, Paige Parrack, Marcellus Banworth, Sheeja Aravindan, Guangpu Li, and Kar-Ming Fung

Abstract

Rab GTPases are essentially molecular switches. They serve as master regulators in intracellular membrane trafficking from the formation and transport of vesicles at the originating organelle to its fusion to the membrane at the target organelle. Their functions are diversified and each has their specific subcellular location. Their expression may vary significantly in the same cell when the level of protein production is significantly different in different physiologic status. One of the best examples is the transition from fetal to mature status of cells. Expression and localization of Rab GTPases in mature and developing brains have not been well studied. Immunohistochemistry is an efficient way in the detection, semiquantitation, and localization of Rab GTPases in tissue sections. It is inexpensive and fast which allow efficient mass screening of many sections. In this chapter, we describe the immunohistochemical assay protocol for analyzing several Rab protein expressions of the Rab5 subfamily, including Rab5, Rab17, Rab22, and Rab31, in developmental (fetal) and mature human brains.

Key words Brain, Neurodevelopment, Fetal brain, Rab5, Rab17, Rab22, Rab31, GTPase

1 Introduction

Rab GTPases are highly conserved molecules and they form the broadest group of GTPases in eukaryotic cells. Rab GTPases are activated by their upstream regulators and the active GTP-bound form activates the downstream effectors. Rab GTPases serve essentially as the traffic controllers and master regulators in intracellular membrane trafficking from the formation and transport of the vesicles from the originating organelles to its target organelles through membrane fusion [1–3]. Their functions are diversified and each has their specific subcellular location.

Sixty-six human Rab GTPases have been identified [4]. There is no surprise that this divergent and highly conserved family of

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molecules play important roles in normal and pathologic conditions. They are involved in many different functions and pathways [5–7]. Rab GTPases have different roles in both physiologic and disease processes in the central nervous system including formation of synaptic vesicles [7], multiple sclerosis [8], and tumor formation [9]. Defective membrane trafficking is associated with neurodegenerative diseases such as Parkinson's disease and Huntington's disease [10].

The Rab5 subfamily members, including Rab5, Rab17, Rab21, Rab22, and Rab31, are involved in endocytosis and endosomal recycling. Rab5 is responsible for early endosome fusion, and is present in plasma membrane, clathrin-coated vesicles, and endosomes [11–13]. Like Rab5, Rab21 is also involved in early endocytosis [14–16]. Rab17 is present in recycling endosomes and is used for transcytosis and postsynaptic trafficking of AMPA and Kainate receptors [17, 18]. Rab22 is for endosomal transport and protein recycling to plasma membrane [19–22]. It is present in early endosomes. Rab31 is used for mannose-6-phosphate receptor transport to endosomes [23] as well as phagocytosis [24].

Elevated Rab31 stabilizes MUC1-C levels in an autoinductive loop that could lead to aberrant signaling and gene expression associated with cancer progression [25].

Defective Rab5 has been shown to impair neuronal migration [26, 27]. Rab3a has been shown to be associated with glioma initiation and progression [28]. However, the in depth functions of different Rab GTPases in the developing brain is yet to be explored [10].

This protocol focuses on the localization of Rab5 subfamily of Rab GTPases, including Rab5, Rab17 (not shown in Fig. 1), Rab22, and Rab31, on tissue section by immunohistochemistry in mature and developing human tissue. Our results [29] showed the general trend that expression of Rab GTPase is high in the late developmental (fetal) stages of human brain but very low in mature human brain with Rab22 as the prototype (Fig. 1).

2 Materials

2.1 Human Autopsy Brain Tissue

Approval from the Institutional Review Board was obtained. Our study materials were limited to brains with no significant histopathologic findings and no significant clinical history of neurologic disorder. The following human brains, including those from the second and third trimesters, were used.

1. Adult brains.
2. Newborn full-term brain.

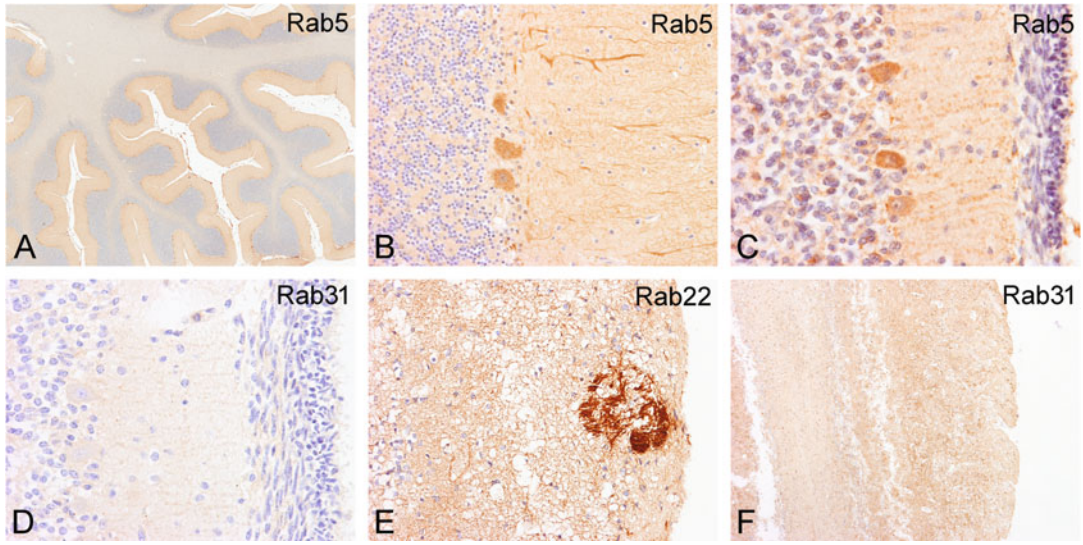

Fig. 1 (**a**, **b**) Expression of Rab5 in mature cerebellum with the expression limited to the cortex, neuropils of the internal granular layer, and cell body (strong expression) in Purkinje cells. (**c**) Expression of Rab5 in fetal cerebellum is similar with the exception that it is not expressed in the external granular layer. (**d**) In contrast, there is no expression of Rab31 in the same fetal brain. (**e**) Rab22 is highly expressed in the glomerulus in olfactory bulb, the surrounding tissue is only weakly expressed. (**f**) In contrast, Rab31 is only weakly expressed in the olfactory bulb

3. Fetal brains from 21 weeks to 33 weeks of gestation.

2.2 Antibodies

Four antibodies were used in this study (*see* **Notes 1** and **2**).

1. Rabbit anti-Rab5 antibody (Cocalico Biologicals, at 1:400 dilution).
2. Rabbit polyclonal anti-Rab17 antibody (GeneTex, at 1:600 dilution).
3. Rabbit EPR9487 anti-Rab22 antibody (Abcam, at 1:100 dilution).
4. Rabbit HPA019717 anti-Rab31 antibody (Sigma, at 1:800 dilution).

Normal human duodenum was used as positive control.

2.3 Staining Materials

1. Leica BOND III automated staining machine (*see* **Notes 1** and **2**).

3 Methods

3.1 Human Brain Samples

3.1.1 Sample Collection

The brains were removed intact during autopsy, fixed in neutral buffered formalin for at least 3 weeks (*see* **Note 3**), and sliced into slabs of about 1 cm think. The targeted areas were dissected out, trimmed to a thickness of about 3 mm and a surface dimension that can fit into the processing cassettes. Cerebral hemispheres were sectioned along the coronal plan, cerebellums were sectioned along the sagittal plane (adults) or horizontal plane (new born and fetal), and the brain stems were sectioned along the horizontal (axial) plane. Representative regions of the central nervous system, including different locations of the telencephalon including the hippocampus, diencephalon, mesencephalon, metencephalon, myelencephalon, and spinal cord were used.

3.1.2 Processing

1. Brain slabs in processing cassettes were infiltrated from formalin through graded alcohol to absolute alcohol and then to xylene followed by paraffin wax in an automated processing machine.
2. The processed tissue slabs were manually embedded into paraffin blocks.
3. Histologic sections were cut at 4μm thick, smoothen out on a warm water bath, attached to positively charged standard sized glass slides, baked at 73 °C for 6 min, and cooled down to room temperature.

3.1.3 Hematoxylin-Eosin Stain

A standard hematoxylin–eosin stained section is prepared for general observation. The slide is stained using a Symphony automated staining machine (Ventana, Tucson, AZ).

3.1.4 Immunohistochemistry (see **Notes 1, 2, 4)**

1. Immunohistochemistry was performed using a Leica-BOND III (Leica Microsystems, Lake County, IL) automated staining machine.
2. The staining process used Leica proprietary chemicals and polymer linked secondary antibodies.
3. Operation was performed as per instruction of the vendor.
4. This system has two antigen retrieval agent based on citrate buffer or EDTA (pH 9.0). The EDTA retrieval program used a heat-induced epitope retrieval (HIER) program. We found that 20-min retrieval using this protocol yielded the best results.
5. The incubation time of primary antibody could be adjusted with this machine and we found 60-min incubation yielded the best results.

6. We used peroxidase-diaminobenzidine as the chromogenic process. Finished slides were lightly counterstained by hematoxylin, dehydrated in graded alcohol, cleared xylene, and sealed with a cover slip using a mounting media.

4 Comments

1. Depending on the fixation, processing protocol, and sensitivity of your immunohistochemistry detection system; the concentration (dilution ratio) of the primary antibodies, antigen retrieval time and protocol, and primary antibody incubation time may need to be adjusted.
2. Sections of duodenum were used to optimize the staining condition for individual antibodies before staining of the brain sections.
3. Due to the variation of many factors in these autopsy brains such as the length of hypoxic period, the post mortem intervals, fixation length, and other factors, there may be variation between individual cases. However, staining results from similar areas of the same case should be similar.

5 Notes

1. The immunohistochemistry protocol that we used was adopted from the protocol for clinical use. There is no specific staining protocol for the Rab antibodies. However, the Leica BOND III and Leica BOND RX that are available to us provide a variety of antigen retrieval protocols, incubation time, and antibody concentration (dilution ratio). We optimize each antibody individually for these three parameters (antigen retrieval protocol, incubation time, and antibody concentration).
2. In our experience, this protocol is very reliable. We understand that an automated staining machine may not be available. Immunohistochemistry can be performed manually. The quality is likely not as outstanding as using an automated staining machine but it is usually acceptable and can be of publication quality.
3. For autopsy brain, they have all been fixed for over 3 weeks and the length of fixation in formation after 3 weeks does not really affect the quality.
4. This protocol can be easily modified for use in different Rab antibodies after appropriate titration of the antibody concentration that fits the instrumentations available in different facilities.

6 Conclusions

Immunoreactivity localized in different regions of the brain with different intensity of staining, depending on the gestational age and maturity of the brain. In general, the level of immunoreactivities of all four Rab GTPases were higher in fetal brain than adult brain and the immunoreactivities diminished in newborn. There were some variations in different anatomic locations and stages of brain development (unpublished data). Rab GTPases, including the Rab5 subfamily, have different roles in both physiologic and disease processes in the central nervous system, including neuronal migration and formation of synaptic vesicles, both of which are essential in development of the central nervous system. Defective Rab GTPases are associated with various neurodegenerative diseases and glioma initiation and progression. However, little is known about the localization of the Rabs in developmental human brains as well as other organs during development and maturation. Use of immunohistochemistry for the Rab5 subfamily of GTPases serves as a good tool for studying localization of the Rab proteins in developmental biology.

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