Supplement 3 (Frosk et al. 2016) – Detailed Discussion on CEP55 antibodies

Objective

To understand the role CEP55 plays in normal brain development. Considering that the mutation in the study cases is predicted to cause truncation after amino acid position 425, a shortened protein product might be detectable with the available antibodies.

Methods

CEP55 protein content was studied by immunoblotting (Western blot) and immunohistochemistry using three commercial antibodies:

Antibody 1: Mouse IgG1 monoclonal anti-CEP55 (clone EMRC10-11-55) was purchased from eBioscience (San Diego CA; catalogue # 14-9809). Full-length human CEP55 recombinant protein was used to immunize BALB/c mice, and spleen cells were fused with NS-1 mouse myeloma cell line to generate the monoclonal antibodies. Antibodies were screened with enzyme linked immunosorbent assay using recombinant CEP55 protein and Western blotting of extracts from cell lines overexpressing the protein and from breast carcinoma samples.[1] However, the published Western blots show only the 55 kDa region (not the entire membrane) so the specificity is not clear. The target epitope has not been mapped (personal communication, Dr. Yoshihiko Hirohashi, December 2014). The antibody was used at 1/250 dilution for immunohistochemistry and 1/800 for immunoblots.

Antibody 2: Rabbit IgG polyclonal anti-CEP55 was purchased from Bioss (Woburn MA; catalogue # bs-7742R). The antibody was created by immunization with a KLH-conjugated synthetic peptide derived from human CEP55. The manufacturer provided the following information about the peptide: "in the range of [amino acid] 155-210/464 … the exact immunogen sequences used to generate these antibodies [are considered] to be proprietary information." The manufacturer's website and datasheet show weak cytoplasmic immunoreactivity in a formalin-fixed / paraffin embedded sample of human breast cancer, but there is no Western blot. The antibody was used at 1/250 dilution for immunohistochemistry and 1/2500 for immunoblots.

Antibody 3: Purified CEP55 MaxPab mouse ascites polyclonal antibody was purchased from Abnova (Taipei, Taiwan; catalogue #H00055165-B01P). The antibody was raised against full-length human CEP55 recombinant protein. According to the manufacturer, the target epitope has not been mapped. The product information sheet shows a clean band at 55 kDa on proteins from a transfected cell line. The antibody was used at 1/500 dilution for immunohistochemistry (no antigen retrieval pretreatment) and 1/100 for immunoblots.

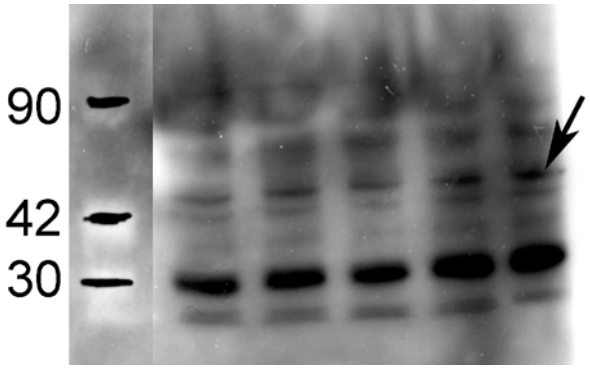
Frontal lobe tissue samples from autopsies were from fetuses (19, 25, and 40 week gestation), a 9-year child, and a 63-year adult. Muscle samples were from one of the fetuses affected by MARCH syndrome and an age-matched fetal control. Frozen tissue samples were homogenized in RIPA buffer and total protein content was determined by the Bradford method. Samples (30 or 50 µg) were boiled for 5 minutes, loaded onto 10% SDS-PAGE gels in 4X SDS loading buffer, and run at 150 V for 90 minutes constant till loading dye reaches at least 1cm to the bottom. Proteins were transferred to PVDF membranes using semi-dry transfer buffer use (BioRad System 170-4155; 25V for 30 min). Membranes were stained with Ponceau S red in 1% glacial acetic acid for 1 min, rinsed in water and

photographed. For the immunoblot, membranes were washed in TBST (20mM Tris-Cl, 500mM NaCl, pH7.5, 0.1% Tween-20) 4 times, then blocked with 5% dry milk in TBST for 1 hr to block the membrane. The primary antibody was diluted in 5% milk in TBST and incubated overnight at 4˚C (dilutions are noted above). Membranes were washed 4 times in TBST, then incubated for 2 hours with the secondary antibody (sheep anti-mouse, Jackson Immunoresearch catalogue # 515-035-062; or goat anti-rabbit, Sigma catalogue # A6154), which was diluted 1:10000 in 5% dry milk in TBST. The membrane was washed again in TBST 4 times and antibodies were detected using Clarity Western ECL Blotting Substrates (Bio-Rad). Films were exposed for 10 to 60 seconds.

Samples for immunostaining included formalin-fixed brain tissue macroarrays (5x5mm samples from 7 brain regions) of autopsy brains from 11 cases (9, 10, 15, 16, 17, 20, 21, 31, and 40 week gestation fetuses along with 2 year and 55 year brains). All were from autopsies conducted at 24-36 weeks postmortem, all have good morphological preservation of tissue including mitotic figures in the germinal tissues, and none had pathological features. Paraffin embedded tissue sections were cut at 5 µm thickness. After dewaxing, endogenous peroxidases were quenched using a 5% hydrogen peroxide in methanol solution for 10 minutes. Antigen retrieval performed by heating slides in 0.01 M sodium citrate buffers at pH 6.0 and 4.0, for the mouse and rabbit Cep55 antibodies respectively, for 20 minutes in a microwave oven. Primary antibodies underwent overnight incubation at 4 °C for all immunostaining. This was followed by incubation with appropriate biotinylated secondary antibodies, followed by reaction with streptavidin-peroxidase, detection with diaminobenzidine (DAB, Sigma D5905), and finally counterstaining with hematoxylin. Negative controls were processed similarly without the primary antibody.

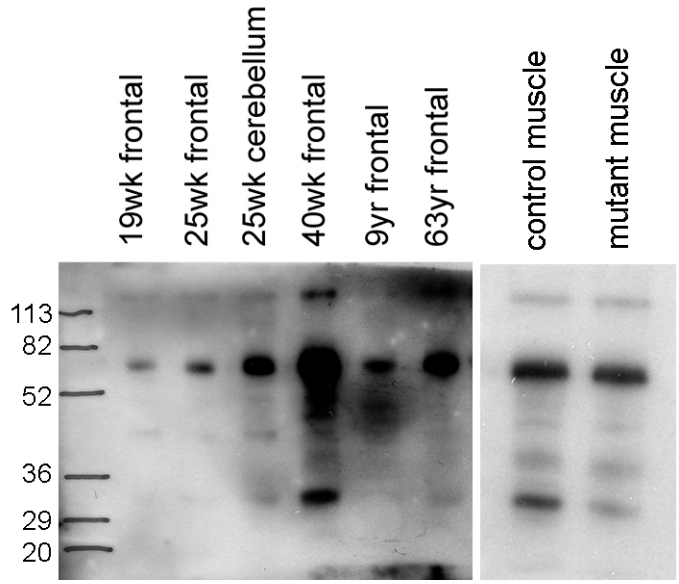
Results

Antibody 1 detected numerous bands including a weak band at approximately 55 kD and a strong band at approximately 30 kD (Supplemental Figure 3.1). We could not get a unique band detection using a variety of different protein loads, blocking conditions, antibody concentrations, and detection times. It was not considered to be reliable for true detection of CEP55.



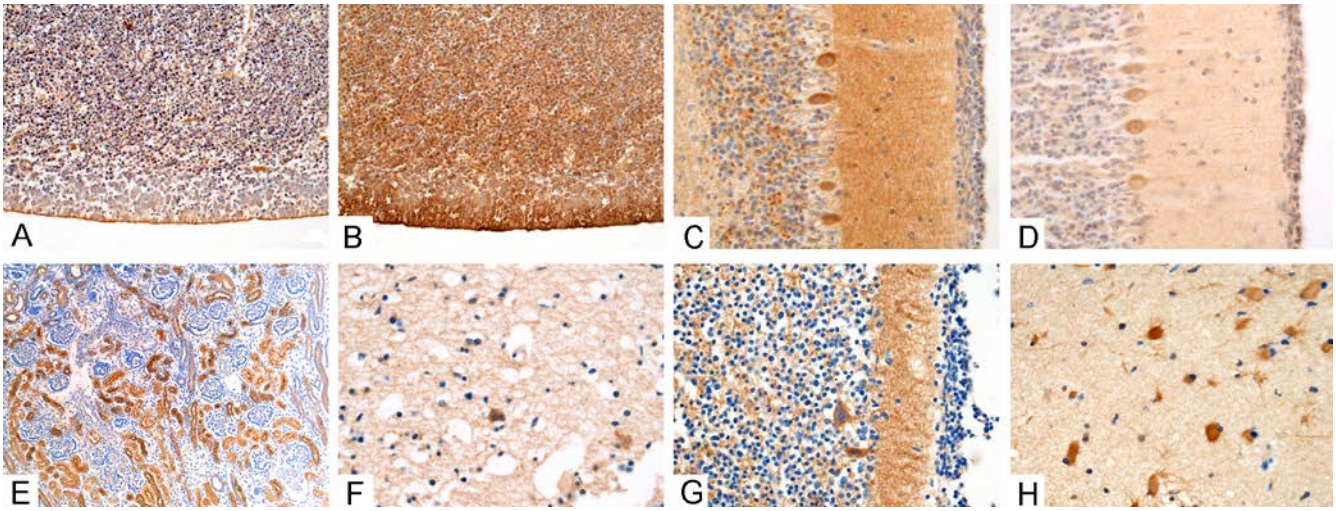
Supplemental Figure 3.1: Western blot showing 5 columns of protein from normal muscle loaded at 10-50 µg per well. Detection was with a mouse monoclonal anti-CEP55 antibody (Antibody 1).

Antibody 2 appeared more promising with a prominent band at approximately 65-70kD on most brain samples. However, multiple bands were detected in the 40 week fetal brain sample, including a strong band at ~30kD. In control muscle samples, the antibody detected bands resembling those of the term brain sample and there was no difference in a sample from one of the affected fetuses (Supplemental Figure 3.2).



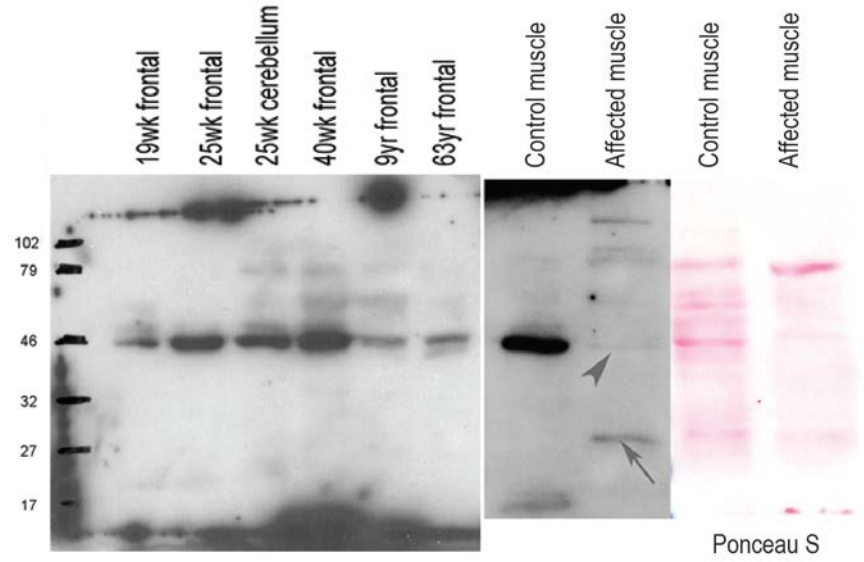
Supplemental Figure 3.2: Western blots showing 6 columns of protein from human brain tissue at various ages and muscle tissue from control and affected fetus. Detection with rabbit polyclonal anti-Cep55 antibody.

Immunohistochemical staining with antibodies 1 and 2 showed very similar results with cytoplasmic labeling in brain and kidney cells at various ages. In the germinal matrix, antibody 2 showed stronger labeling than antibody 1. The converse was true in mature tissues. However, there was no punctate intracellular labeling in proliferative cell populations, as would be predicted from the published cell culture experiments involving CEP55 (see main text, Figure 5). Furthermore, there was no difference in labeling pattern between control and CEP55 mutant samples and unexpectedly, multinucleate neurons exhibited strong labeling (Supplemental Figure 3.3). Omission of the primary antibody was associated with no labeling. We concluded that neither of the two antibodies actually detect CEP55 in human tissue.



Supplemental Figure 3.3: Immunohistochemical detection using antibodies 1 and 2. (A) Ganglionic eminence from 17-week gestation fetus labeled using the antibody 1; (B) Same tissue labeled using antibody 2; (C) Cerebellum from a 2-week infant who was born at full term labeled using antibody 1; (D) Same tissue labeled using antibody 2; (E) Kidney from a 29-week gestation fetus showing labeling in the tubule cells using antibody 1. (F) Cytoplasmic labeling of a multinucleate neuron in the temporal cortex of the 32-week gestation affected fetus using antibody 1. (G) Cellular labeling in the cerebellum of the 35-week gestation affected fetus using antibody 1. (H) Cellular labeling in the temporal cortex of the 35-week gestation affected fetus using antibody 2. Original magnifications: 200x – E; 400x- A, B; 600x – all others.

Antibody 3 yielded a fairly clean single band in the region of 46kD and faint bands at higher molecular weights in some of the normal brain samples. The intensity of the band was greatest at 25 and 40 weeks gestation. A similar band was present in control muscle, but not in muscle from one of the affected fetuses (Supplemental Figure 3.4). The Western blot result was consistent with the prediction about the normal developing brain and the mutant tissue. This same antibody was used to reliably detect overexpressed CEP55 in our cell culture experiments (main text, Figure 5). Although the apparent molecular weight is not quite that expected in these human tissue experiments, the absence of the major band in the mutant does suggest it may be the right protein.



Supplemental Figure 3.4: Western blots showing 6 columns of protein from human brain tissue at various ages and muscle tissue from control and affected fetus. In the affected fetus, the major band is lost (arrowhead) and a new minor band is present (arrow) although Ponceau S staining does show a degradation in the affected compared to control. Detection with mouse polyclonal anti-Cep55 antibody.

Using brain tissue samples with and without two different antigen retrieval methods (heated citrate buffer at pH 6; heated Tris-EDTA at pH 9) we could not get the antibody 3 to convincingly label anything. In particular, we searched for punctate labeling in proliferating cell populations of the germinal matrix, external granular zone of the cerebellum, and the internal lamina of the dentate gyrus.

Conclusions

Only one of the three antibodies tested (mouse ascites polyclonal CEP55 antibody from Abnova; catalogue #H00055165-B01P) gives human tissue Western blot results the can potentially be attributed to CEP55 detection. The other two (mouse monoclonal and rabbit polyclonal) had Western blot and immunohistochemical results that do not make sense in the context of known structure and function of CEP55.[2] Our finding is particularly concerning because the mouse monoclonal antibody has been used to provide evidence for CEP55 having a role in breast cancer.[1,3]

Several limitations must be pointed out. Although numerous anti-CEP55 antibodies are commercially available, most are not reported to be useable for immunohistochemistry. The specific protein epitopes have not been identified for any of the antibodies. We did not perform any blocking experiments to try to determine either the specific correct targeting of the mouse polyclonal antibody, or the incorrect detection of the other two antibodies as this was outside of the scope of this study. In addition, no frozen brain or kidney tissue was available from the mutant cases. Our Western blot analysis was confined to muscle, and it is not clear how relevant this tissue is given the features of MARCH syndrome.

References

[1] Inoda, S., et al. Cep55/c10orf3, a tumor antigen derived from a centrosome residing protein in breast carcinoma. Journal of immunotherapy 32, 474-485 (2009).

[2] Fabbro, M., et al. Cdk1/Erk2- and Plk1-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. Developmental cell 9, 477-488 (2005).

[3] Jeffery, J., Sinha, D., Srihari, S., Kalimutho, M. & Khanna, K.K. Beyond cytokinesis: the emerging roles of CEP55 in tumorigenesis. Oncogene 35, 683-690 (2016).