Grant agreement no. 769051

EpiCARE – a network for rare and complex epilepsies

HP-ERN-2016 European Reference Networks / Framework Partnership Agreement

D6.1 Neuropathology standards

Work Package: 6

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Dissemination Level		
PU	Public	PU
CO	Confidential, only for members of the consortium (including the Commission Services)	
CI	Classified, as referred to in Commission Decision 2001/844/EC	

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1. Version log

Version	Date	Released by	Nature of Change
1	25/01/18	I.Blumcke UKER	1st version

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2. Definition and acronyms

Acronyms	Definitions
EEBB	European Epilepsy Brain Bank
ILAE	International League against Epilepsy

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3. Introduction

Workpackage 6 (E-neuropathology) promotes a European Tissue Bank and Reference Pathology Service for the histological and molecular-genetic analysis of human surgical specimens, which will be accessible to all EpiCARE partners. The core tools for this is the EU-FP6/FP7 funded European Epilepsy Brain Bank (EEBB) a standardized collection of 9523 epilepsy surgery patients from 36 centers in 12 European countries (Blumcke et al. 2017). EEBB was designed as a virtual database using the web-based SecuTrial platform collecting a minimal data set for each, double-encoded patient, e.g. histopathological diagnosis, age at epilepsy surgery, age at seizure onset, sex, location of the epileptic lesion, year of surgery, 12 month postsurgical outcome with or without further drug prescription.

Two deliverables were instrumental for this goal: to establish international guidelines for histopathologic diagnosis of epilepsy surgery specimens (**D6.1**) and to create high quality tissue biospecimens for clinically-applicable molecular analysis that can be also transfered amongst partner institutions (D6.2).

E-neuropathology will offer the large experience in standardized neuropathological examination protocols (i.e. ILAE guidelines and classification systems), tissue preservation and banking to all partner of EpiCARE.

Our guidelines for tissue fixation and storage (long-term preservation) as well as snap frozen tissue acquisition will be standardized as SOP and distributed to all participants.

4. Activities carried out and results

Activities carried out

Previous work of the commission on Diagnostic Methods and Task Force for Neuropathology of the ILAE was instrumental to develop international recommendations for the neuropathologic work-up of epilepsy surgery human brain samples. These recommendations were developed during a 3 year discussion at international conferences together with a panel of distinguished international experts selected by the ILAE. Results of this work were published following peer-review in the scientific journal Epilepsia (Blumcke et al. 2016).

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Results

• Systematic tissue sampling of 3-5 mm slabs along a defined anatomical axis and application of a limited immunohistochemical panel will ensure reliable differential diagnosis of main pathologies encountered in epilepsy surgery. Use photographic documentation (with ruler) to enable co-registration with MRI or EEG finding.
• Adapt recommended fixation and staining protocols to own laboratory environment, including a panel of antibodies for validation of specific brain lesions (see technical annex IV). Application of antibodies other than recommended requires scientific validation process.
• Allow long-term storage of native tissue at -80°C for further molecular-diagnostic use and research. Selection of tissue for long-term storage should follow specified tissue procurement protocol and photographic documentation.
• Prepare a comprehensive histopathology report referring to anatomical landmarks and orientation and clear statement of the histopathological diagnosis according to current classification systems. Relevant auxiliary clinical and diagnostic information including MR imaging findings should be included into the section "clinical history".

Technical Annex I: SOP for tissue inspection, distribution and processing in epilepsy surgery (from Blümcke et al. 2016)

1.) Anatomically intact tissue samples are recommended for histopathological assessment. The resected surgical specimen(s) should be procured in the operation room by the local neuropathology laboratory. Neurosurgeons could label the anterior-posterior or dorsal-ventral axis of each sample with staples or ink. Suspected lesions or other regions of interest should also be marked, such as the site of an epileptic focus determined by presurgical or intraoperative electrophysiological recordings.

2.) At the neuropathology laboratory, document the specimen weight in grams (specify pre- or postfixation) and size of tissue specimen (preferably by a photograph that includes a metric ruler next to the tissue). Weights of ultrasonic surgical aspirates also provide estimates of total volume of brain tissue removed.

3.) If snap frozen tissue will be required for molecular-biological investigations, dissect fresh tissue specimen with 3-5 mm interval parallel slices according to the anatomical orientation (preferably at coronal planes along anterior-posterior axis). Label all slices and document their order by photography, using an alphabetical or numerical system (e.g. I, II, III, ... 1,2,3, ... A,B,C,... a,b,c,). Labeling margins with different colored inks may be helpful in some cases. If possible, slices containing a macroscopic abnormality or the center of the epileptogenic zone as defined by imaging and/or electrophysiology should be labeled as such. Tissue from this region should be apportioned for histology or banking (snap frozen, formalin or paraformaldehyde fixed, cell culture, electron microscopy, etc.) as determined

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appropriate in each case and dictated by size of lesion. The neuropathologist needs to make judgments with small samples in the allocation of tissue for research to ensure there is no compromise to the histological diagnosis. The remaining sections can be alternatively fixed (for histology) or frozen (for tissue banking at -80°C). Use appropriate vials to store fresh frozen samples and label vials with de-identifying study number (preferably lab-number), slice number (see #5) and date of storage.

4.) If snap frozen tissue is not required, the specimen should be immersed in fixative overnight (10% formalin or 4% paraformaldehyde for at least 12h) and subsequently dissected into 3-5 mm interval parallel slices according to anatomical orientation (preferably at coronal planes along anterior-posterior axis). Label all slices and document their order by photography, using an alphabetical or numerical system. Depending on the size of the specimen alternate slices will be saved for histopathology and embedded into paraffin to allow thin cutting at 4-7 µm for routine histology, histochemical stains as well as antibody immunoreactivities. If possible, the slice containing a macroscopic abnormality or capturing the center of the epileptogenic zone as defined by imaging and/or electrophysiology should be labeled as such. The remaining tissue blocks will become available for tissue banking and/or other research projects.

5.) Slabs from the resection border should be labeled and separately embedded into paraffin for systematic histopathological examination.

Technical Annex II: SOP for tissue processing and storage protocols (from Blümcke et al. 2016)

It is recommended to use a standardized paraffin embedding protocol using commercially available semi- or fully automated equipment.

Paraffin embedded tissue specimens should be cut with a rotatory microtome at 4 - 7 µm thickness (preferably at 4 µm). Blocs should be cooled to -15°C before cutting. Sections should be stretched in heated water bath at 40°C before mounting on coated glass slides. Allow drying for 30 min at 60°C or overnight at 36°C.

For tissue bio-banking, unfixed tissue should be snap-frozen in isopentane (2-methylbutane) cooled to the temperature of liquid nitrogen and stored at -80°C in appropriate tissue container. Tissue covering in compound cryostat embedding medium will prevent drying artifacts occurring during long-term storage.

Technical Annex III: SOP for a standard immunohistochemistry protocol for paraffin-embedded sections (can be modified according to local requirements; from Blümcke et al. 2016)

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1) De-paraffinize (de-wax) sections in xylene 2 x 10 min; hydrate with 100% isopropanol 5 min.; hydrate with 96% isopropanol 5 min.; hydrate with 70% isopropanol 5 min.; rinse in distilled water

2) Antigen retrieval to unmask antigenic determinants: boil slides for 2 x 10 min in citrate buffer (e.g. microwave). Refill buffer after first round; cool for at least 10 min and rinse 2-3 times in Tris buffered solution (TBS)

3) Block endogenous peroxidase activity: Inactivate endogenous peroxidase by covering tissue with 3% hydrogen peroxide for 15 min (45ml methanol + 5ml H2O2 (30%)) and rinse 2-3 times in TBS

4) Preventing non-specific staining: Blocking in 3% fetal calf serum/ 1% goat serum/ 0.1% triton X 100 in TBS for 1h. Do not rinse! Carefully wipe away excess serum around the sections with tissue paper and apply primary antibody diluted in blocking solution. Incubate over night at 4°C

5) Visualize bound antibodies (as recommended in Table 2): rinse 2-3 times in TBS; apply biotinylated secondary antibodies and incubate for 10 min; Rinse 2-3 times in TBS; apply streptavidin peroxidase and incubate for 10 min; rinse 2-3 times in TBS; Apply DAB chromogen mixture to tissue section for 5-10 min (visual control of immunoreactivity); rinse in distilled water; counterstain in haematoxylin. (2-5 sec); rinse in water; dehydrate samples (70% isopropanol, 96% isopropanol, 100% isopropanol, xylene, 10 dips per rinse); mount coverslip using appropriate medium (e.g. permount).

Technical Annex IV: Recommended antibodies for the diagnosis of epilepsy associated brain lesions (from Blümcke et al. 2016)

Staining	HS	MCD	Tumors	Vasc.	Infl.	Scars	No lesion
HE	x	x	x	x	x	x	x
CV-LFB	x	x	x	x	x	x	x
GFAP	x	x	x	x	x	x	x
MAP2		x	x				x
NeuN	x	x	x	x	x	x	x
NFL		x					x
Vim		x					x
CD34		x	x				x
Ki67			x				x

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IDH1			x				x
CD68			x		x	x	x
CD45	x3				x	x	x

Legend to table: HE – haematoxylin-eosin; CV-LFB – cresyl violet-luxol fast blue; GFAP – glial fibrillary acidic protein; MAP2 – microtubule associated protein 2 (clone HM2); NeuN – Neuronal nuclei (clone A60); NFL – neurofilament protein (clone SMI32); VIM – vimentin; CD34 – oncofetal class II epitope CD34 (clone QBenD10); KI67 – proliferation marker (clone Mib1); IDH1 - R132H point mutation specific antibody; CD68 – antibody specific for macrophages and microglia; CD45 – antibody specific for lymphocytes; HS – hippocampal sclerosis; MCD – malformations of cortical development; Vasc. – vascular malformations including cavernomas and arterio-venous malformations; Infl. – inflammation; no lesion – refers to histopathological specimens in which none of the aforementioned principle histopathological categories can be identified. This will usually require a more generous application of immunohistochemical investigations.

5 Conclusions

We provided standardized protocols, terminology use and guidelines for a cost-effective diagnosis of epileptogenic brain lesions. Standardized neuropathological work-up of tissue procurement, handling and processing is also paramount to the implementation of evidence-based medicine, e.g. by supporting clinical trials. Further efforts in dissemination across European countries include the use of a collaborative virtual microscopy platform and regular teaching courses, e.g. 26.-29.7.2018 in Erlangen, Germany; see EpiCare and ILAE websites.

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