

## Is autopsy tissue a valid control for epilepsy surgery tissue in microRNA studies?

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### SUMMARY



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**MicroRNAs (miRNAs) are differentially expressed in the brain under pathologic conditions and may therefore represent both therapeutic targets and diagnostic or prognostic biomarkers for neurologic diseases, including epilepsy. In fact, miRNA expression profiles have been investigated in the hippocampi of patients with epilepsy in comparison with control, nonepileptic cases. Unfortunately, the interpretation of these data is difficult because surgically resected epileptic tissue is generally compared with control tissue obtained from autopsies. To challenge the validity of this approach, we performed an miRNA microarray on the laser microdissected granule cell layer of the human hippocampus obtained from surgical samples of patients with epilepsy, autptic nonepileptic controls, and patients with autptic epilepsy, using the latter as internal control. Unfortunately, it is extremely difficult to collect autopsy material from documented epilepsy individuals who died of non–epilepsy-related causes—we found only two such cases. However, hierarchical clustering of all samples showed that those obtained from autopsies of patients with epilepsy segregated with the other autptic samples (controls) and not with the bioptic tissues from the surgery patients, suggesting that the origin of the tissue (surgery or autopsy) may be prevalent over the underlying pathology (epilepsy or not epilepsy). Even taking into account the limitations due to the small number of cases, this observation arises concerns on the use of autopsy tissue as control for this kind of studies.**

**KEY WORDS:** microRNAs, Microarray, Epilepsy.

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MicroRNAs (miRNAs), are small, endogenous, noncoding RNAs that regulate gene expression at the posttranscriptional level by inhibiting mRNA translation. About 70% of the known miRNAs are expressed in the brain, and many are specific to neurons.<sup>1</sup> It has been hypothesized that miRNAs might actively participate in the pathogenesis of central nervous system (CNS) diseases; their expression levels have been investigated in many neurologic disorders, including Alzheimer disease, Parkinson disease,<sup>2,3</sup> and temporal lobe epilepsy (TLE).<sup>4–8</sup>

miRNAs may represent novel therapeutic targets for these diseases. For example, the silencing of miR-134 has been demonstrated to exert prolonged seizure-suppressant and neuroprotective actions in a murine model of epilepsy.<sup>9</sup> Moreover, miRNAs may serve as disease biomarkers.<sup>10,11</sup> With reference to epilepsy, studies in rodents found a correlation between specific miRNA expression patterns and the phase of the disease.<sup>4,6,7</sup> If confirmed in humans, these

patterns may help to predict patients who will develop epilepsy after a potentially epileptogenic insult. Moreover, analysis of miRNA expression in surgical samples may help in the prognostic evaluation of postsurgical epilepsy.<sup>5</sup>

miRNA expression profiles have been also investigated in the hippocampus of patients with TLE in comparison with control, nonepileptic cases.<sup>12</sup> These studies<sup>12</sup> raise the following concerns: (1) the use of heterogeneous brain areas like the hippocampus may influence the miRNA expression levels compared to healthy cases, because the cellular composition of an epileptic brain is dramatically changed compared with the normal tissue (e.g., neuronal loss, astrogliosis, and microgliosis); (2) the interpretation of these data is difficult also because the control tissue used to investigate miRNA expression levels was from autopsies, whereas the epileptic tissue was from surgical specimens, and it is questionable if autopsy samples are valid controls for surgical epilepsy samples.

Therefore, the aim of this study was to challenge the validity of this approach. First, we strongly attenuated the confound of cell heterogeneity by performing an miRNA microarray on the laser microdissected granule cell layer of the hippocampus, a nearly homogeneous preparation. Second, we compared bioptic (surgical) samples of TLE patients, autoptic (nonepileptic) controls, and autoptic epileptic patients, using these latter as an “internal control” for the hierarchical cluster analysis. The assumption was that, if autopsy controls were valid, the autopsy epilepsy group would segregate with the surgery epilepsy group. If instead the controls were not valid, that is, if the postmortem condition was prevalent over the disease condition, the autopsy epilepsy group would segregate with the autopsy control group. Unfortunately, it is difficult to obtain autopsy material from documented epilepsy individuals who died because of non-epilepsy-related causes, and we managed to find only two such cases.

## MATERIALS AND METHODS

### Patients and controls

The present study was approved by the Ethics Committee of Bologna (Comitato Etico Indipendente dell’Azienda USL della Città di Bologna). Surgical hippocampal samples from 12 drug-resistant TLE patients (3 male and 9 female) were collected at the Epilepsy Surgery Center of the IRCCS Institute of Neurological Sciences of Bologna (Table 1).

Epileptologic evaluation, wakefulness/sleep electroencephalography (EEG), and continuous long-term video-EEG monitoring for seizure recording were performed on all epileptic patients before surgery. Ictal clinical and EEG semiology and electroclinical correlations were used to identify the epileptogenic area. Brain magnetic resonance imaging (MRI) and computed tomography (CT) scan (when necessary) were also used for proper identification of the epileptogenic focus. The main surgical specimens

(hippocampus and/or temporal pole) were removed “en bloc” and spatially oriented to allow a proper histopathologic examination. All the methods were carried out in accordance with the approved guidelines, and samples were handled in compliance with the Helsinki declaration (<http://www.wma.net/en/30publications/10policies/b3/>).

Autoptic hippocampal tissue was from two epileptic patients (both female) who died of lung pathologies and 10 patients (three male and seven female) without history of epilepsy or seizures (Table 1). All these samples were from the tissue bank of the Bellaria Hospital in Bologna and our institutional review board or ethics committee waived the need for informed consent.

### Histology and microdissection

After collection, tissues obtained from both surgeries and autopsies were immediately immersed in formalin for 48–72 h, and then paraffin embedded. Ten-micron-thick sections were cut and mounted on slides for laser microdissection (LMD). Samples were de-waxed, washed in ethanol, and stained with hematoxylin. Hippocampal samples from autoptic cases did not display signs of hypoxia, for example, red degeneration features such as pronounced cytoplasmic eosinophilia, collapse of cytoplasm with accentuated (artifactual) pericellular spaces, or pyknotic nuclei with indistinct nucleoli. In addition, the cerebellar granular layer did not present autolytic swelling or status spongiosus.<sup>13</sup>

Laser microdissection was then performed essentially as described previously.<sup>5</sup> Briefly, slides were positioned in a stencil laser (SL) microcut/microtest dissector (Nikon, Tokyo, Japan). The intensity, aperture, and cutting velocity were calibrated to obtain the sharpest cut, and the pulsed ultraviolet (UV) laser beam was directed along the borders of the dentate gyrus granule cell layer (GCL). To obtain an adequate amount of tissue for RNA purification, the microdissected GCL from at least three to four slices per patient was pooled together. Total RNA was extracted using an RNA purification kit (RecoverAll Total Nucleic Acid Isolation kit; Life Technologies, CA, U.S.A.).

The concentration and the quality of the RNA were investigated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.) and an Agilent 2100 Bioanalyzer. Formalin-fixed, paraffin-embedded, laser-microdissected samples had an RNA Integrity Number (RIN) <5 (in a scale 1–10), as expected based on previous reports.<sup>14</sup> This would prevent accurate analysis of mRNAs, but not of miRNA.<sup>14</sup> Moreover, to increase accuracy, we verified that samples had a pick containing miRNAs (20–25 nucleotides) in the electropherograms obtained by the Bioanalyzer.

### Hierarchical clustering

One hundred nanograms of total RNA per sample was employed for microarray analysis (Human micro-RNA

Table 1. Cases included in the study

Patient number	Group	Gender	Age at surgery	Age at death	Epileptogenic insult	Years after diagnosis	Seizures per month	Drug therapy	Postmortem delay (h)	Cause of death
01	Epilepsy, surgery	M	60		None identified	38	>30	VPA, CBZ, TGB		
02	Epilepsy, surgery	F	50		None identified	38	5-12	CBZ, TPM, PB		
03	Epilepsy, surgery	M	44		None identified	12	3-8	TPM, LVT		
04	Epilepsy, surgery	M	36		None identified	7	8-10	LVT, PB, CLB		
05	Epilepsy, surgery	F	47		None identified	33	2-3	PB, CBZ		
06	Epilepsy, surgery	F	33		Febrile convulsions	30	4-5	TPM, CBZ, VPA, PB		
07	Epilepsy, surgery	F	31		Febrile convulsions	18	3-8	LVT, CBZ, CLB		
08	Epilepsy, surgery	F	31		Febrile convulsions	10	8-12	PB, TPM		
09	Epilepsy, surgery	F	33		Febrile convulsions	24	3-4	LTG, LVT, PB		
10	Epilepsy, surgery	F	32		Febrile convulsions	27	4-10	CBZ		
11	Epilepsy, surgery	F	32		Febrile convulsions	20	9-10	OXC, LVT		
12	Epilepsy, surgery	F	37		Febrile convulsions	35	7-10	CBZ, TPM		
13	Epilepsy, autopsy	F		70	Trauma	48	4-30		44	Pneumonia
14	Epilepsy, autopsy	F		46	None identified	4	25-30		26	Pulmonary edema
15	Control, autopsy	M		40					38	Cerebral infarction
16	Control, autopsy	F		47					35	Myocardial infarction
17	Control, autopsy	F		51					26	Pulmonary edema
18	Control, autopsy	F		34					34	Pneumonia
19	Control, autopsy	M		38					24	Myocardial infarction
20	Control, autopsy	F		48					25	Cerebral infarction
21	Control, autopsy	F		44					28	Pulmonary thromboembolism
22	Control, autopsy	F		57					55	Pulmonary thromboembolism
23	Control, autopsy	F		50					42	Pulmonary edema
24	Control, autopsy	M		56					20	Pulmonary thromboembolism

CBZ, carbamazepine; CLB, clobazam; LTG, lamotrigine; LVT, levetiracetam; OXC, oxcarbazepine; PB, pentobarbital; TGB, tiagabine; TPM, topiramate; VPA, valproic acid.

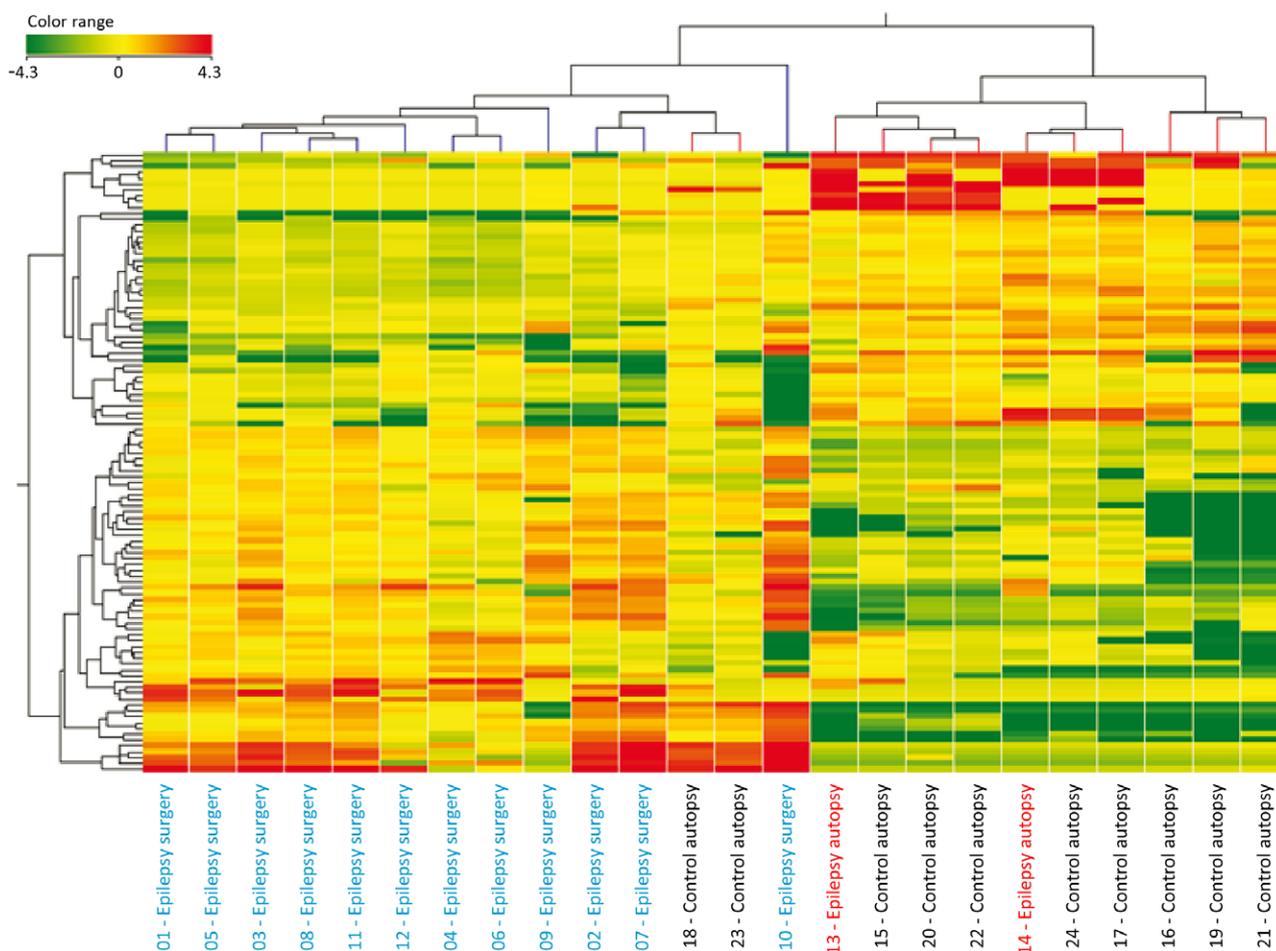
Microarray V3, #G4470C, Agilent Technologies, Santa Clara, CA, U.S.A.). The chip allowed simultaneous analysis of 1,200 human miRNAs obtained from the Sanger miR-BASE database (Release 10.1). RNA labeling and hybridization were performed according to the manufacturer's indications. Row data were obtained using an Agilent scanner and the Feature Extraction 10.5 software (Agilent Technologies).

The GeneSpring GX 12 software (Agilent Technologies) was employed to analyze microarray results. All negative values were transformed at 1.0, followed by Quantile normalization and log<sub>2</sub> transformation. Differentially expressed miRNAs were identified by comparing autoptic controls with autoptic and bioptic epileptic samples, applying a twofold-change filter, the Mann-Whitney test, and Benjamini-Hochberg correction (adjusted  $p < 0.05$ ). Manhattan correlation was used for cluster analysis.

## RESULTS

### Subjects

Surgical specimens from 12 patients with TLE indicated in numbers in Table 1 were laser-microdissected and employed for microarray analysis. Neuropathologic examination evidenced that all these patients had type 1 hippocampal sclerosis.<sup>15</sup> This group was composed of three men and nine women, with mean age at surgery of 39 (31–60), mean years after epilepsy diagnosis of 24 (7–38), and approximately 10 seizures per month before surgery (2 to >30). The two autoptic TLE specimens (Table 1, cases 13 and 14) also had type 1 hippocampal sclerosis. They were from two female patients who died for lung diseases (pulmonary edema, pneumonia). Their mean age at death was 58 (range 46–70), mean years after epilepsy diagnosis was 26 (4–48), and they had 4–30 seizures/month.



**Figure 1.**

Cluster analysis of miRNAs differentially expressed in the granule cell layer (GCL) in TLE patients (including two autoptic epileptic cases) and in the control group. Each column represents an individual case and each row represents one miRNA. Colors represent the miRNA expression level in each sample, referred to miRNA average expression: higher-red, lower-green. Differentially expressed miRNAs were identified applying a twofold-change filter, the Mann-Whitney test, and Benjamini-Hochberg correction (adjusted  $p < 0.05$ ).

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Control specimens were from 10 nonepileptic individuals listed in Table 1 (cases 15–24). This group was composed of three men and seven women with mean age at death of 46 (34–57), who died of lung or heart diseases (pulmonary edema, pneumonia, pulmonary thromboembolism, or myocardial infarction) except for cases 15 and 20, who died of cerebral infarction.

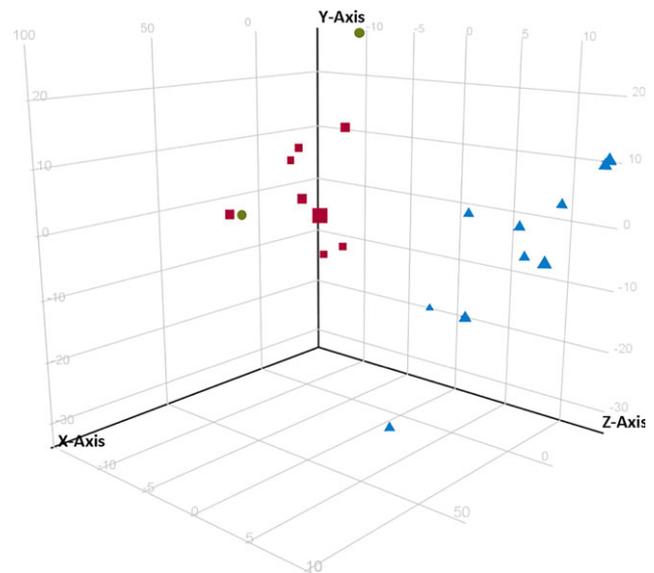
### Microarray

We investigated miRNA expression in the laser-microdissected granule cell layer (GCL) from the three groups: surgical specimens of epileptic patients, autoptic samples of cases without history of epilepsy, and autoptic samples from TLE patients. After comparing epileptic (both autoptic and bioptic) and control samples, we detected significant changes in the expression levels of 106 miRNAs. Hierarchical clustering of all samples showed two main clusters: all surgical epilepsy samples segregated together in one cluster, and all autoptic control cases in the other, with the exception of two cases (nos.18 and 23) that segregated at the margin of the surgery group, adjacent to the other autopsy cases. One surgery case (no. 10) displayed a unique profile in that, even if belonging to the larger surgery cluster, segregated independently. In summary, with few minor exceptions, we obtained a very good separation between epilepsy surgery and control autopsy cases. However, contrary to the expectation, the two samples obtained from autopsies of TLE patients segregated with the control group of only autoptic samples (Fig. 1).

An unsupervised principal component analysis (PCA) plot of the data is shown in Figure 2. The analysis was performed using the expression of all human miRNAs, without any feature selection. Therefore, samples are grouped according to their global miRNA expression profile. A clear separation between autoptic and surgery samples can be observed.

## DISCUSSION

Previous studies have explored differences between miRNA expression patterns in surgical epileptic tissue compared with autoptic control tissue.<sup>12</sup> However, the possible bias generated by the utilization of postmortem tissue was not evaluated in depth. This study aimed at addressing this specific issue, by introducing in the comparison a third group, that is, autopsy specimens from individuals with epilepsy. We acknowledge that a limitation of the present study is the very limited number of cases in this group (two only). Unfortunately, it is difficult to obtain autopsy tissue from patients with a documented history of epilepsy, who died of non-epilepsy-related causes. Even if it would have been desirable to have a larger cohort of such cases, and even taking into account the considerations described in the Results section, the segregation of these two cases with the other



**Figure 2.**

Principal component analysis (PCA) plot. Scatter plot showing the samples coordinates on the three main principal components. Autopsy controls are represented as red squares; autopsy epilepsy are green circles, and surgery epilepsy cases are blue triangles. *Epilepsia Open* © ILAE

autopsy, nonepileptic ones (and not with the surgical epileptic, as expected) is very clear. A different pathology beyond the source of tissue may affect miRNA signatures, because like most autoptic control cases, two epileptic autoptic cases died from lung diseases. In any event, this unexpected finding seems to depend on postmortem modifications and not on sample processing, because all samples (either autoptic or bioptic) were processed in the same manner. Specifically, we were careful that the following parameters were identical in all samples: (1) the time between tissue resection and formalin fixation, (2) the laser microdissection procedure, and (3) the RNA quality for miRNA detection. Although these may not completely exclude a contribution of minor differences in tissue processing, it seems fair to say that the observation poses serious concerns on the use of autopsy controls for this kind of study.

A possible approach that may be proposed to maintain the use of control autopsy cases in this kind of study, thereby integrating the limited number of epilepsy autopsy cases with the much more readily available surgical ones, may be to first run cluster analysis of microarray data to compare autoptic epileptic with autoptic controls, and to then perform a further hierarchical clustering on all samples (including surgical samples) based on those miRNAs identified as differentially expressed in the first step. We explored this possibility with our samples, and observed significant changes in the expression levels of 36 miRNAs between autoptic epileptic and autoptic controls. When cluster analysis has been run including all groups, the two autoptic epileptic

cases segregated with the other epileptic and not with the other autopsies (data not shown). Although this experiment must be viewed as preliminary, being based on the only two autoptic epilepsy cases, it appears to be a more accurate approach than the surgery–epilepsy versus autopsy–control approach. Nonetheless, further analysis with increased cohorts of autoptic TLE cases will be needed to unequivocally identify TLE-dependent miRNA expression changes.

In conclusion, microarray platforms can provide important insights in the search for novel biomarkers or therapeutic targets, but limitations in the experimental approach must be carefully considered. Even if based on a number of cases too limited to provide conclusive evidence, the present results indicate that the postmortem modifications may have greater impact than the disease background on the data generated, with a potentially serious hindrance in their interpretation.

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## ADDITIONAL CONTRIBUTORS

PR and MS designed the study and wrote the manuscript. PR and SZ performed the experiments. MF performed bioinformatics analyses. MG, GM, RM, and GR prepared the human samples.

## DISCLOSURE

None of the authors has any conflict of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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