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Characterization of gene expression profiling of mouse tissues obtained during the postmortem interval

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ABSTRACT

Attempts to establish a tissue bank from autopsy samples have led to uncovering of the secrets of many diseases. Here, we examined the length of time that the RNA from postmortem tissues is available for microarray analysis and reported the gene expression profile for up- and down-regulated genes during the postmortem interval. We extracted RNA from fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) brains and livers of three different groups of mice: 1) mice immediately after death, 2) mice that were stored at room temperature for 3 h after death, and 3) mice that were stored at 4 °C for 18 h after death, as this storage resembles the human autopsy process in Japan. The RNA quality of the brain and the liver was maintained up to 18 h during the postmortem interval. Based on the microarray analysis, we selected genes that were altered by >1.3-fold or <0.77-fold and classified these genes using hierarchical cluster analysis following DAVID gene ontology analysis. These studies revealed that cytoskeleton-related genes were enriched in the set of up-regulated genes, while serine protease inhibitors were enriched in the set of down-regulated genes. Interestingly, although the RNA quality was maintained due to high RNA integrity number (RIN) values, up-regulated genes were not validated by quantitative PCR, suggesting that these genes may become fragmented or modified by an unknown mechanism. Taken together, our findings suggest that under typical autopsy conditions, gene expression profiles that reflect disease pathology can be examined by understanding comprehensive recognition of postmortem fluctuation of gene expression.

1. Introduction

During the past decade, tissue banks of autopsy tissues (i.e., DNA, RNA, and protein from fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissues) have been established and have helped elucidate disease pathology as well as development of new diagnostic techniques (Ferrer et al., 2008; Kretzschmar, 2009). Among the tissues pooled in the tissue bank, brain tissue is especially useful for the pathological analysis of neuropsychiatric and neurodegenerative disorders because other biopsy samples are limited (Ferrer et al., 2008; Kretzschmar, 2009). Indeed, for Alzheimer's disease (Ginsberg et al., 2006), Parkinson's disease (Greene, 2012), Huntington's disease (Cha, 2007), schizophrenia (Benes, 2010; Horvath et al., 2011), and bipolar disorder (Ginsberg et al., 2012), microarray analysis has revealed disease pathogenesis from postmortem brain samples from autopsy cases. Therefore, storage of higher quality samples in tissue banks will enable important pathological studies, especially when using novel and more accurate techniques, such as mass spectrometry (Ho Kim et al., 2015; Tagawa et al., 2015), RNAseq (Zhao et al., 2014), and miRNA analysis (Eisele et al., 2012; Wang et al., 2008).

Because RNA is remarkably unstable compared to the other specimens stored in the autopsy tissue bank, techniques to obtain good quality RNA from postmortem specimens are crucial. These techniques are even more critical because microarray and RNAseq techniques are advancing and have become increasingly used for gene expression analysis. RNA quality is strongly correlated with the RNA integrity number (RIN) values that are calculated using the Agilent Bioanalyzer (Kap et al., 2014), and thus, numerous studies have investigated the factors that may affect the RIN value in RNA extracted from postmortem specimens (Atz et al., 2007; Chevyreva et al., 2008; Papapetropoulos et al., 2007; Stan et al., 2006; Weis et al., 2007). These studies have revealed that RNA quality is affected by cerebrospinal fluid pH, the length and severity of the agonal state, the antemortem hypoxic state, and the length of the postmortem interval before autopsy (Bauer, 2007; Catts et al., 2005). In forensic medicine, the postmortem changes of biomolecules have attracted considerable attention in order to accurately ascertain

Abbreviations: DEG, differentially expressed gene; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; HIF, hypoxic inducible factor; RIN, RNA integrity number; RMA, robust multiarray average.

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the time of death (Bauer, 2007; Vennemann and Koppelkamm, 2010). Actually, the decay of RNA collected from cadavers has also been investigated to estimate the postmortem interval (Bauer et al., 2003; Hansen et al., 2014; van Doorn et al., 2011). Furthermore, gene expression profiles may be modified by hypoxia and other factors induced by somatic death (Durrenberger et al., 2010). Therefore, when postmortem-derived RNA is used for pathological molecular analyses, such as microarray and RNAseq analysis, understanding of the common postmortem modifications in the gene expression profile in each organ is crucial to correctly interpret any findings.

Because many recent reports suggest that RNA from FFPE samples is valuable for microarray analysis, several commercial companies have developed kits to extract this RNA (Klopfleisch et al., 2011). Many FFPE specimens collected from biopsy and autopsy materials are stored in the pathology laboratory; thus, microarray and other RNA analyses from these FFPE samples may lead to a better understanding of disease pathology. Indeed, previous clinical studies demonstrated the utility of microarray analysis using RNA from FFPE samples (Budczies et al., 2011; Frank et al., 2007; Jacobson et al., 2011; Roberts et al., 2007; Sadi et al., 2011); however, studies which have demonstrated a thorough comparison of results from microarray between RNA derived from FFPE and that derived from FF samples are still limited (Abdueva et al., 2010; April et al., 2009; Mittempergher et al., 2011; Thomas et al., 2013).

In this study, we first compared the RNA quality of mouse brain and liver harvested in the same postmortem interval as that of typical human autopsies in Japan. We next compared sequential gene expression profiles by microarray for postmortem brain and liver and identified gene clusters that were commonly altered in both brain and liver during the postmortem interval. Finally, using microarray analysis, we compared gene expression profiles between FF and FFPE samples obtained at the same postmortem interval. These results provide important information about microarray analysis of postmortem samples from autopsy cases.

2. Materials and methods

2.1. Animals and experimental design

Pathological autopsies are usually performed approximately 2–3 h after death during the daytime or the next morning after a nighttime death (Buesa et al., 2004). Before autopsy examination, the cadavers are usually stored at room temperature during the daytime or at 4 °C overnight. Therefore, RNA was extracted from FF brains and livers from mice under three different conditions to resemble the human autopsy process: 1) mice immediately after death, as a control; 2) mice that were stored at room temperature for 3 h after death; and 3) mice that were stored at 4 °C for 18 h after death.

Then, we extracted RNA from these frozen brain and liver samples and confirmed their RNA quality. Furthermore, we performed microarray analysis to examine changes in the gene expression profiles that occur during the postmortem interval.

After typical autopsy, FFPE tissue blocks are usually created using specimens collected from eviscerated organs. Therefore, we also created FFPE tissue blocks at the same time using mouse organs obtained under the three conditions described above. We then purified RNA from the FFPE tissue blocks and then performed microarray analysis on this RNA. We next compared gene expression profiles between FF and FFPE samples at three different postmortem times, as described for the FF samples.

Our experimental design is summarized in Fig. 1. For these experiments, we purchased 10-week-old male BALB/c mice from SLC (Hamamatsu, Japan). All experimental procedures and protocols were approved by the Animal Care and Use Committee of Chubu University and conformed to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. RNA isolation from FF and FFPE tissues and estimation of RNA integrity

Excised livers and brains were first immersed in RNALater (Ambion, Austin, TX, USA) and cryopreserved (FF samples). Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) from these FF samples. In addition to FF samples, we fixed excised livers and brains with 10% formalin for 24 h and then embedded in paraffin (FFPE samples). FFPE samples were stored at room temperature for 7 d and were then cut into 10-μm wide slices, followed by extraction of RNA using RNeasy FFPE Kit (Qiagen). RIN values of the extracted RNA were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.3. Microarray experiments and data analysis

Comparative analysis of gene expression profiles was performed using the MG-430 PM Array Strips and the Affymetrix GeneAtlas microarray system (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. For RNA samples from FF tissues, the corresponding amplified and biotin-labeled antisense RNA (aRNA) was synthesized using a GeneChip 3′IVT Express Kit (Affymetrix). The resulting aRNA was fragmented as described by the manufacturer. On the other hand, for RNA samples from FFPE tissues, the corresponding amplified and biotin-labeled DNA fragments were generated using the WT-Ovation FFPE RNA Amplification System V2 (NuGEN, San Carlos, CA, USA) followed by the Encore Biotin Module (NuGEN) according to the manufacturers' protocols.

The biotin-labeled aRNAs from FF tissues and fragmented DNA from FFPE tissues were hybridized with the array strips at 45 °C for 16 h. After hybridization, the strips were washed and stained in the GeneAtlas Fluidics Station using the GeneAtlas Hybridization, Wash, and Stain Kit (Affymetrix), and the intensity of each hybridized probe was measured using the GeneAtlas Imaging Station.

The obtained CEL files were normalized and summarized using the robust multiarray average (RMA) method with Expression Console software (Affymetrix). The results were comparatively analyzed and visualized using the Subio platform (Subio, Amami, Japan). Differentially

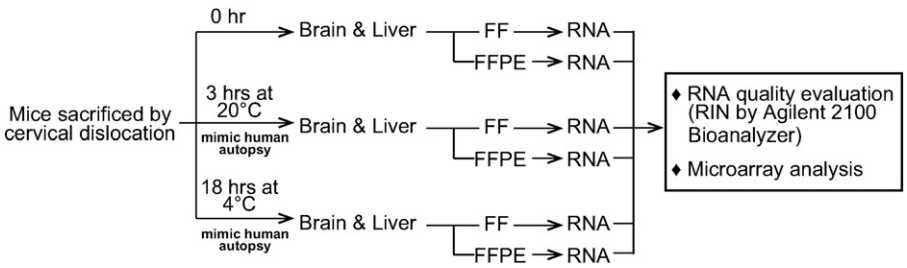

Fig. 1. Overview of experimental design. RNA was extracted from fresh-frozen (FF) or formalin-fixed paraffin-embedded (FFPE) samples derived from mouse brain and liver under three different conditions: 1) immediately after death as a control, 2) after storage at room temperature for 3 h after death, and 3) after storage at 4 °C for 18 h after death, a condition that resembles the human autopsy process. After RNA qualities were estimated using the Agilent 2100 Bioanalyzer, microarray analysis was performed.