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Inter-age variability of *bona fide* unvaried transcripts Normalization of quantitative PCR data in ischemic stroke

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Abstract

Background: Aging is a major risk factor for a variety of neurobiological diseases leading to variations of transcriptional expression in affected tissues. Reverse transcription of RNA followed by quantitative PCR is a powerful technique for detection and quantification of specific transcripts differentially expressed. An essential prerequisite for accurate interpretation of quantitative PCR data obtained from expression studies is an appropriate normalization process. Therefore we validated the expression of the most frequently used reference genes consisting of *Gapdh* and *Actb* as well as *Hmbs*, *Hprt1* and *Gusb* in an animal model of mice in respect to two major influence factors, aging and ischemia. In the experimental settings we intended to reflect variations in both, the local and systemic immune response.
Results: The consistency in gene expression of the tested transcripts was quantified based on standard deviation, correlation analysis and two algorithms available as Visual Basic Applications (VBA) termed *GeNorm* and *Normfinder*. Overall, the results of the study proofed the suitability of *Actb* in combination with *Gapdh* and with tissue-specific limitations *Hmbs* in brain and *Gusb* in white blood cells as the most stable transcripts for accurate normalization. We clearly demonstrated that both, the aging process *per se* and aging in combination with ischemia are confounding factors with respect to the expression stability of *Hprt1*.
Conclusions: The present study emphasizes the urgent need to validate the expression stability also from *bona fide* unvaried transcripts under specific conditions of investigation to ensure adequate normalization of qPCR data. Based on the expression stability, the use of *Gapdh* and *Actb* as highly abundant transcripts for normalization of qPCR data under conditions of aging and ischemia in a mouse model was evaluated. However, for low abundant genes the use of *Hmbs* in brain and *Gusb* in white blood cells is recommended.

Keywords: Aging; Inflammation; Ischemia; Brain; White blood cells; Reference genes; Housekeeping; Real-time RT-PCR; qPCR; *Gapdh*; *Actb*; *Hmbs*; *Gusb*; *Hprt1*

1. Introduction

Aging is a major risk factor for a variety of neurobiological diseases such as stroke, dementia, Parkinson's and Alzheimer's disease, leading to severe impairment of cognitive and motor skills. Besides direct damage of the nervous system, stroke also influences the tightly balanced interaction between the nervous and immune system. Suppression of the immune system after stroke is a common phenomenon in these patients increasing the susceptibility for infections finally resulting in a systemic inflammatory response, which is the most relevant complication (Meisel et al., 2005). Cerebrovascular events are responsible for about 10% of death worldwide (Rosamond et al., 2007). About 700,000 people in the US and approximately 1.1 million in Europe sustain an initial or recurrent stroke per year (Wolfe et al., 2000; Truelsen et al., 2005; Truelsen et al., 2006; Rosamond et al., 2007). Stroke is the leading cause of death, ranking behind cardiovascular and heart disease, infectious diseases and cancer and one of the prevailing factors of long-term disability

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(Carmichael, 2005; Truelsen et al., 2006; Rosamond et al., 2007). In line with that, studies on this age-related disease will be essential for combating the sequelae of stroke.

In aging research most of the clinical studies are performed in an observational manner on humans or in experimental settings in rodents, whereas especially mouse models exhibit advantages due to the availability of genetic knockouts. However, in studies focusing on age-related diseases, the age of enrolled animals is often restricted to the period of adolescence, thus there are concerns about the generalization of the obtained results to the entire lifespan. For studying specific transcripts differentially expressed in age-related diseases, reverse transcription followed by quantitative PCR (qPCR) is a powerful technique.

Recent studies suggest that normal brain aging is associated with functional alteration and variations in the gene expression profile (Lee et al., 2000). To analyze expression profiles by qPCR, several variables need to be extensively controlled. To date, the most frequently used strategy for normalization is internal standards, termed reference or control genes (Bustin, 2002; Huggett et al., 2005). It is strongly recommended to normalize qPCR data against a panel of reference genes (probably two or three) whose expression stability has been shown to be stable under the conditions of investigation (Bustin, 2002). Surprisingly the most frequently used reference genes in neurobiological research are glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and beta-actin (*Actb*) in spite of the fact, that there are concerns about their regulation (Harrison et al., 2000; Suzuki et al., 2000). Particular *Gapdh* as one of the most frequently used reference gene shows no constant expression under various experimental conditions (for review see Suzuki et al., 2000). Especially in age-induced apoptosis of cultured neurons or following focal ischemia in rat brain there is evidence that *Gapdh* is differentially expressed (Ishitani et al., 1996; Tanaka et al., 2002). In contrast, there are numerous reports that expression of *Gapdh* remains constant under ischemic conditions, *e.g.* after permanent MCAO in rats, whereas *Actb* has been shown to vary in the corresponding mRNA level (Harrison et al., 2000). According to these studies, the assumption of constantly expressed reference genes under various conditions or in different tissues is discussed controversially. At present, there are some interesting statistical approaches to identify the most stable expressed control genes from a set of candidates under various conditions. With the assumption that control genes are not co-regulated, the *GeNorm* algorithm determines the two most stable expressed genes by stepwise exclusion of the worst scoring (Vandesompele et al., 2002). The *Normfinder* algorithm calculates the variation of the candidate reference genes in a side-by-side comparison and shows less sensitivity towards co-regulations (Andersen et al., 2004).

In the present study we tested the commonly used reference genes *Gapdh* and *Actb* as well as hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), hydroxymethylbilane synthase (*Hmbs*) and glucuronidase beta (*Gusb*) as potential control genes for two of the strongest influence factors, aging *per se* and ischemia in combination with ageing concerning local inflammatory reaction (brain) and systemic immune response (white blood cells) to evaluate reference transcripts valuable for normalization processes in functional studies.

2. Materials and methods

2.1. Aged animal and model of stroke

The study was carried out on male C57BL/6 mice of distinct age and approved by the local authorities (Thueringer Landesamt, Weimar, Germany). The C57BL/6 mice strain has an average lifespan of ~26 months (Forster et al., 2003). All mice were maintained in a specific pathogen-free environment at room temperature (22 °C) at 68% humidity and 12:12 h light/dark cycles with access to water and food *ad libitum* and were tested negative for parasites and other routine pathogens using sentinel mice held under identical conditions. Investigation of age-related effects has been carried out on mice *(native)* randomly assigned to 2 months (juvenile), 9 months (adult), 15 months (senior) and 24 months (aged), with five mice per group, thereby meets the requirements by Coleman et al. (2004). To study the effect of ischemia dependent on aging another 64 animals were equally divided into four groups (2, 9, 15 and 24 months) and randomly assigned to intervention (MCAO, sham) with two reperfusion times (2 days and 7 days).

Middle cerebral artery occlusion (MCAO) has been introduced by Koizumi et al. (1986) and Longa et al. (1989) for rats and subsequently modified for mice by Belayev (Koizumi et al., 1986; Longa et al., 1989; Belayev et al., 1999). Here we applied this method to occlude the middle cerebral artery (MCA) for 30 min. Briefly, mice were anesthetized with 2.5% isoflorane in a N_2O:O_2 (3:1) mixture. Through a middle neck incision the right common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were carefully dissected from surrounding nerves and fascia. A 7-0 nylon monofilament (70SPRe, Doccol Corp, USA) coated with silicone rubber on the tip (0.2 ± 0.01 mm diameter) was introduced into the ICA through an incision of the right CCA up to the circle of Willis. In this position the suture occluded MCA. During the MCA occlusion body temperature was maintained at physiological level using a heating pad. After 30 min of occlusion the suture was withdrawn to restore the blood flow. Sham animals underwent anesthesia and surgical procedure like the treatment group with exception of middle cerebral artery occlusion.

2.2. Sample preparation

Under deep anesthesia (isoflurane) blood samples were taken *via* heart puncture and stabilized in PAXgene Blood