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The relationship between reproductive and biochemical ageing at the time of the menopausal transition

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ABSTRACT

The biochemical ageing status of women in the menopausal transition was studied using quantitative analysis of age- and autophagy-related gene activities (CDC42 and MAP1LC3 genes were selected as target genes).

Free estradiol and progesterone levels in saliva were estimated. General linear models were used to determine the relationship between lifestyle, health status, socioeconomic factors and CDC42 and MAP1LC3 gene expression levels.

Gene expression analysis revealed (1) an increasing expression of CDC42 gene after 45 years in women, (2) expression level of CDC42 gene associated with menopausal status, (3) while endocrine status was found to associate with the expression of both of the studied age-related genes, (4) the "never used hormonal contraceptives" and "obese nutritional status" were the strongest factors for increased level of age-related gene expressions, and (5) changes in gene expression levels by ageing should be studied by considering not only chronological, but also biological ages.

Gene expression profile of ageing has mostly been studied in model systems or human blood samples, but rarely in human saliva samples. The concordance of results between the present and former gene expression analyses, and the simplicity of saliva sample collection emphasizes the importance of saliva tissue samples in gene expression analyses especially in epidemiological surveys.

1. Introduction

Ageing is a progressive functional decline with age, ultimately culminating in mortality, including a decline in physiological functional capacity (to maintain the baseline homeostasis) and in hormonal activity, a decline in fitness, an increase in vulnerability, an irreversible age-related process of loss of viability and a decrease in fecundity (Lopez-Otin et al., 2013; Partridge and Mangel, 1999). This functional decline is also accompanied by a considerable change of body structure. Body dimensions and proportions, the absolute and relative mass of body components, as well as the structure of tissues change gradually and moderately until the age of 60–70 years and very intensively thereafter (the onset and the velocity of these changes show very high variation both within and between populations (Baumgartner, 2009; D'Antona et al., 2003; de Magalhaes, 2004; te Velde and Pearson, 2002; Zsakai et al., 2016)).

Contrary to the strictly ordered developmental processes of human ontogenesis, the order of the human ageing events and processes cannot be explained yet (Kowald and Kirkwood, 1996; de Magalhaes and Toussaint, 2004). Whether the first sign of ageing is damage to the cardio-vascular system or that of the central nervous system, the changes in the metabolic system or in the cell division — it cannot be predicted on the basis of actual knowledge, because (1) these processes have very high individual variability and (2) all factors that can influence the ageing processes, have not yet been identified, and (3) even the already identified factors have been mostly studied independently.

As in the case of any other biological feature of the human body, the biological ageing processes are controlled by genetic background and influenced by environmental factors as well as by the interactions between these two. The genetic regulation and the biochemical mechanisms behind human ageing have not yet been fully explored. Numerous pathways modulating ageing have been identified in model organisms, but there is a debate whether ageing is a consequence of a genetically programmed process (Christensen et al., 2006; Kenyon, 2010; Tacutu et al., 2012; Wheeler and Kim, 2011). It is known that (1) the accumulation of late acting deleterious mutations, (2) changes in hormonal signaling in the life span, (3) the decrease in resting metabolic rate, (4) the shortening of chromosomal telomeres, (5) the numerical or

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functional decline in tissue stem cells, (6) the rate of ageing regulated by a nutrient-sensitive signaling and (7) the accumulation of damages to the molecules are the main accompanying mechanisms of the ageing process (Burtner et al., 2009; Calderwood et al., 2009; Hsieh and Yamane, 2008; de Magalhaes, 2004; Weinert and Timiras, 2003).

The general ageing process includes the reproductive senescence in both sexes due to an age-related decline in steroid production, but the pace of human reproductive senescence shows considerable sexual dimorphism. The gradual deterioration of the male reproductive function begins in the late 30s and continues until death. By contrast, the first signs of the decline in the female reproductive function can also be observed in the late 30s, but menopause, when the female reproductive function ceases, occurs around the age of 50 (Broekmans et al., 2007; Hansen et al., 2008). The menopausal transition has a very important role in the ageing process, since the declining ovarian function results not only in the loss of fertility but also in metabolic, physiological and body-structural changes of the ageing female body (Lebrun et al., 2006; Poehlman, 2002; Toth et al., 2000; Zsakai et al., 2016).

The present study aimed at analyzing the biochemical ageing status of women in the menopausal transition by quantitative analysis of age-related gene activities. It has been previously hypothesized in the 'mitochondrial-lysosomal axis theory of ageing' (Brunk and Terman, 2002) that the insufficient and declining rate of autophagic turnover – by damaging cellular components and normal metabolism – decreases the survival of cells, i.e. the declining level of autophagic activity has been assumed to be one of the main reasons of ageing.

Therefore, the target genes CDC42 (cell division cycle 42) and MAP1LC3 (microtubule-associated protein 1 light chain 3 beta) were selected from age-related and autophagy-related genes. The autophagic activity level was assessed by estimating the gene expression level of MAP1LC3 gene, an autophagy-related gene (a phagophore or autophagosome marker). The MAP1LC3 protein is a subunit of neuronal microtubule-associated MAP1A and MAP1B proteins that are involved in microtubule assembly and important for neurogenesis. Cytosolic LC3-I is conjugated to phosphatidylethanolamine to become phagophore- or autophagosome-associated LC3-II.269. The LC3 family includes LC3A, LC3B, LC3B2 and LC3C. These proteins are involved in the biogenesis of autophagosomes and in cargo recruitment (Klionsky et al., 2016). Therefore the MAP1LC3 gene activity plays an important role in autophagy, a process that involves the bulk degradation of cytoplasmic component (He et al., 2003). Studies on Atg8/LC3 homologs implicate an important role for this gene in autophagy in every model system (Kerber et al., 2009; Wang et al., 2007).

Since natural aging in mice has been confirmed to relate to increased CDC42 gene expression (Wang et al., 2007), and CDC42 showed a strongly increased expression with age in human adults (age 57–97 (Kerber et al., 2009)), the CDC42 gene was chosen to analyse the level of cellular senescence in a group of Hungarian females. The CDC42 protein is a GTPase member of the Rho-subfamily. This family of proteins regulates signaling pathways of cell morphology, migration, endocytosis and cell cycle progression. The CDC42 protein regulates actin polymerization through its direct binding to Neural Wiskott-Aldrich syndrome protein (N-WASP, (Kerber et al., 2009; Park et al., 2015)). The pattern in CDC42 and MAP1LC3 gene expression by age and by reproductive ageing was analysed, furthermore the relationship between gene expression levels of the studied age-related genes and biometric, lifestyle and socioeconomic factors were also analysed.

2. Material and methods

2.1. Subjects

After anthropometric, body composition and osteometric examinations, subjects in the Hungarian Menopause Study (2011–2015, n: 1747, aged 27 to 84 years) were interviewed using structured questionnaires about their reproductive and menstrual history, socio-demographic background, lifestyle, health conditions and subjective health. The studied subgroup of women in the gene expression level estimation consisted of 42 subjects (Table 1). Saliva sample collection was chosen as a possible alternative to blood sample collection as a source of human RNA for gene expression analysis in epidemiological studies. The study of the quality and quantity of extracted salivary RNA was the initial aim of the present analysis. Saliva was collected from 100 subjects, but only 51 samples (9 samples for gene selection, 42 samples for the final gene expression study) were selected for cDNA synthesis, the selection was based on the RNA parameters, see below. The initial subsample of 100 women in the biochemical ageing analysis were randomly selected from the sample of the Hungarian Menopause Study, and 9 samples of the 51 appropriate samples were randomly selected from the 3 age-groups for gene selection.

Table 1
Distribution of women by age and menopausal status in the gene expression analysis.

Age-groups (years)	n	%	Menopausal status categories	n	%
1: 26–45	14	33.3	Premenopausal	14	33.3
2: 46–65	17	40.5	Perimenopausal	13	31.0
3: 66–85	11	26.2	Postmenopausal	15	35.7
Total	42	100.0	Total	42	100.0

2.2. Saliva collection

Saliva samples were collected and preserved with the Oragene RNA self-collection kit, according to the manufacturer's instructions (RE-100; DNA Genotek Inc., Ontario, Canada) in 2015. Women were asked to avoid eating, drinking, chewing gum or brushing their teeth for 30 min before sampling. Saliva samples were collected between 10.00 A.M. and 12.00 (noon) over a 5 min period. Exactly 2 ml was collected from each subject, and every saliva sample was extensively shaken for at least 60 s to mix saliva with the 2 ml of Oragene RNA stabilizing liquid (stored in the cap of the collection tube). Samples were immediately placed in ice for subsequent RNA isolation (every sample was frozen for at least one night). The samples were stored for a maximum of 2 weeks at − 20 °C before RNA isolation.

2.3. Salivary RNA extraction and contaminating DNA removal

Total salivary RNA was extracted and treated to exclude contaminating genomic DNA by using a protocol combining the Oragene RNA self-collection kit (RE-100; DNA Genotek Inc., Ontario, Canada), the RNeasy Micro kit (Qiagen, Hilden, Germany) and the TURBO DNase kit (Life Technologies, Carlsbad, CA, USA).

In brief, 2 ml of whole saliva was mixed with 2 ml of Oragene RNA liquid and incubated for 1 h at 50 °C. 1 ml of the mix was heated at 90 °C for 15 min and allowed to cool to room temperature. The incubation was performed on two separate tubes for each sample (2 × 500 μl), but the parallels were added onto the same RNeasy MinElute Spin Column during the RNA extraction phase. Then 20 μl of the Oragene RNA neutralizer solution was added to the samples. Samples were mixed, incubated on ice for 10 min, and centrifuged at 13 000 × *g* for 3 min at room temperature. The supernatant was collected and the lysis step followed by dissolving the supernatant in 350 μl RLT buffer (RNeasy Micro kit) solution. Thereafter, 350 μl of 70% ethanol was added to the solution. After mixing the solution, the samples were transferred to RNeasy MinElute spin columns. The columns were centrifuged for 15 s at 8000 × *g*, then 350 μl of RW1 buffer (RNeasy Micro kit) was added to the columns, and they were centrifuged again for 15 s at 8000 × *g*. Afterwards, 500 μl of RPE buffer (RNeasy Micro kit) was added to the columns and were centrifuged for 15 s at 8000 × *g*. 500 μl of 80% ethanol was added and the columns were centrifuged for 2 min at 8000 × *g*, after which they were placed