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IMMUNOHISTOCHEMICAL SEARCH FOR AGE-RELATED SENESCENT CELLS IN HUMAN ENDOMETRIUM

DIMITAR PARVANOV*, NINA VIDOLOVA, RUMIANA GANEVA, GEORGI STAMENOV, TODOR CHAUSHEV

Nadezhda, Women's Health Hospital, 3 "Blaga vest" Street, Sofia, Bulgaria
**Corresponding author: dimparvanov@abv.bg*

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Abstract: Forty five endometrial tissue sections from forty five patients with repeated implantation failure (RIF) were studied. All biopsies were taken in spontaneous cycle during the expected window of implantation (7-9 days after ovulation). Tissues were immunostained with antibodies against β-galactosidase, histone H2AX, nuclear high-mobility group box 1 (HMGB1), $p16^{ink4a}$ and p21. Expression of β-galactosidase, HMGB1 and H2AX was observed in a relatively high percentage of endometrial cells (92%, 58% and 39%, respectively). In contrast, p16 and p21 were positively stained in particular single cells in the endometrial stroma, glandular and surface epithelia. The p21 immunoreactivity occurred in 4.7% of the gland cells while p16 staining was observed in 9.8% of the endometrial gland cells. The overall percentage of positive p21 and p16 staining of the stromal cells was 0.6% and 3.5%, respectively. Correlation analysis revealed that the patient's age correlated significantly only with the percentage of p21-stained cells in endometrial stroma and glands (R=0.53; p=0.002 and R=0.54; p=0.001, respectively). The obtained results show that β-galactosidase, H2AX, HMGB1 and p16 are expressed, not only by age-senescent cells but also by other cell types in endometrial tissue. Among the studied markers the most appropriate and easy detectable immunohistochemical biomarker for cellular age-associated senescence in human endometrium is p21.

INTRODUCTION

Senescence is a condition, observed in normal cells, which approach the limit of their reproductive potential. This specific process is characterized by a flat and large cellular morphology and irreversible proliferative arrest (de Jesus and Blasco, 2012). The obtained stabilized apoptotic cells are determined as senescent or so called "zombie" cells (Oropesa-Ávila *et al.,* 2014). Telomere shortening is

established as one of the major mechanisms, leading to age-related senescence (Olovnikov, 1973; Hayflick, 1997). It is also known that similar condition, called stress-induced premature senescence can be reached through other non-telomeric metabolic pathways with the participation of cytokines, oncogenes and environmental stress factors (Toussaint *et al.,* 2000; Serrano and Blasco, 2001). Both of these senescence types act through the modulation of determined group of proteins including p53 and Rb (Collado and Blasco, 2007). The observed phosphorylation and stabilization of p53 during this process of telomere shortening activate cyclin-dependent kinase inhibitor $p21^{CIP1}$ (Choudhury *et al.,* 2007). In contrast, stress-induced senescence is functioning mainly through the activation of another key molecule – $p16^{INK4a}$ (p16) (Serrano *et al.,* 1996). Both p21 and p16 activation leads to a similar response that includes the inhibition of Rb and inactivation of E2F transcription factor (Collado and Blasco, 2007).

The identification of appropriate biomarkers for *in vivo* detection and quantification of senescent cells is still a challenging task (Sharpless and Sherr, 2015). Although the measurement of β-galactosidase remains one of the most frequently applied biomarkers for cell senescence (Debacq-Chainiaux *et al.,* 2009), often a non-specific activity can disturb the obtained results (Going et al., 2002). Cell cycle regulators such as $p16^{INK4a}$, $p21^{CIP1}$ and p53 are also commonly applied to detect senescent cells (Collado and Serrano, 2010). Persistent DNA damage is a critical trigger of cellular age-related senescence and could be identified by the presence and quantity of g-H2A-X (D'Adda Di Fagagna *et al.,* 2003). High mobility group box-1 (HMGB1) is another biomarker for cellular senescence. It belongs to the family of alarmins, which is an inflammatory mediator, important in tissue damage signalling (Yamada and Maruyama, 2007). In senescent cells, HMGB1 translocates from the nucleus to the cytoplasm and the extracellular space, facilitating the release of IL-1b, IL-6 and other cytokines and chemokines (Davalos *et al.,* 2013; Biran *et al.,* 2017). Although HMGB1 is used as a biomarker to detect senescent cells in various tissues (Davalos *et al.,* 2013; Wiley *et al.,* 2016; Biran *et al.,* 2017), its utility to detect senescent cells in human endometrium remains to be investigated.

Recent studies showed that senescent cells are present in different types of tissues, including human endometrium (Brighton *et al.,* 2017). It could be suggested that they play an important role in the process of tissue modification during the window of implantation (Brighton *et al.,* 2017). However, data about senescence cells in the endometrium and appropriate imunohistochemical (IHC) markers for their detection are still scarce.

In this study, we examined and compared the stromal and glandular epithelial β-galactosidase, H2AX, HMGB1, $p16^{ink4a}$ and p21 expression in the functional endometrial layer of women in different age.

MATERIALS AND METHODS

Subjects and Tissue collection

Forty-five women were enrolled in the study. All participants provided written informed consent in accordance with the guidelines of the Declaration of Helsinki, 2000. The patients underwent a standard endometrial biopsy in spontaneous cycle by pipelle suction between days 21 and 24 of the menstrual cycle based on urinary luteinizing hormone (LH) surge detection (LH +7 to LH +10). LH surge was measured by LH detection kit (Sepal Inc., Boston, MA). The endometrial tissue was subjected to paraffin embedding for immunochistochemical analysis.

Immunohistochemistry

Immunohistochemical staining was performed on the paraffin sections by the method using a Novolink Polymer Detection System (*Leica* Biosystems, Wetzlar, Hesse, Germany) and antibodies, listed in (**Table 1.**).

In brief, each section was deparaffinized in xylene and rehydrated with graded alcohols. Subsequently, the sections were placed in 0.01 M citrate buffer (pH 6.0). Next, 3% hydrogen peroxide was applied to block endogenous peroxidase activity and then the tissue was incubated in 0.4% Casein in phosphate-buffered saline to reduce nonspecific binding of primary antibody and polymer. The sections were incubated with appropriate antibody listed in Table 1 (diluted with antibody IHC diluent Novocastra (RE7133, *Leica* Biosystems, Wetzlar, Hesse, Germany). After that samples/slides were treated with Novocastra Postprimary Block, containing 10% (v/v) animal serum in tris-buffered saline, to enhance penetration of the subsequent polymer reagent. Consequently, poly-HRP anti-mouse/rabbit IgG reagent (NovoLink Polymer) containing 10% (v/v) animal serum in tris-buffered saline was applied to localize the primary antibody, and the reaction product was visualized by incubation with the substrate/chromogen, 3,3′-diaminobenzidine (DAB) prepared from Novocastra DAB Chromogen and NovoLink DAB Substrate Buffer (Polymer), as a brown precipitate. Finally, the sections were counterstained with Novocastra Hematoxylin (0.02%).

Positive staining was identified by brown-colored products in the nucleus and/or in the cytoplasm. Nuclear and cytoplasmic staining in each section was evaluated by the percentage of stained cells.

Table 1. Antibodies and preconditioning applied for immunohistochemistry

Antibody designation	Source	Identifiers
β-galactosidase, GLB1	Elabscience	E-AB-34238
H2AX	Elabscience	E-AB-21330
HMGB1	Elabscience	E-AB-12505
P16^{INK4a}	Elabscience	E-AB-13142
p21	Elabscience	E-AB-32448

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 21.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze all data. Spearman's correlation coefficient was used to assess the strength of the linear relationship between the percentage of positively stained cells and women age. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

As shown in Figure 1, high expression of β-galactosidase, HMGB1 and H2AX was observed in normal endometrial stroma and the endo¬metrial glandular epithelial cells (**Fig. 1**). Relatively high percentage of endometrial cells had detectable expression of β-galactosidase, HMGB1 and H2AX with mean values of 92%, 58% and 39%, respectively. The other two biomarkers (p16 and p21) were positively stained in particular single cells in the endometrial stroma, glandular, and surface epithelia. It could be suggested that these characteristics make them more suitable for precise analysis.

The p21 immunoreactivity occurred in 4.7% (1.2 – 12.6%) of the gland cells while p16 staining was observed in 9.8% (3.2 – 16.1%) of the endometrial gland cells. The overall percentage of positive p21 and p16 staining of the stromal cells was lower - 0.6% (0 – 2.1%) and 3.5% (0.4 – 12%), accordingly.

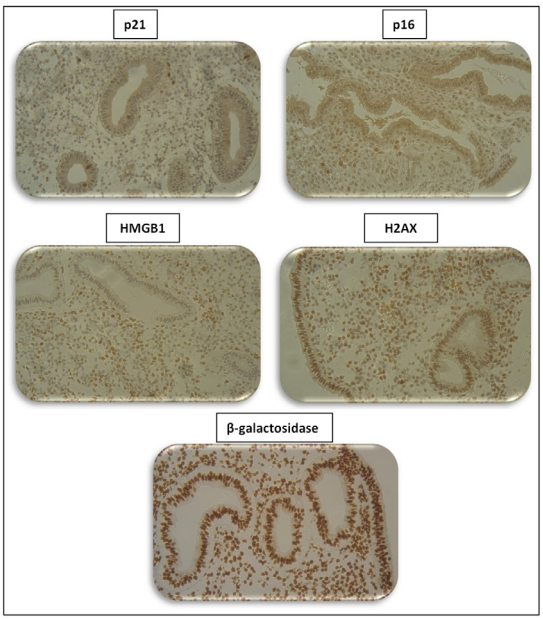

Fig. 1 . Endometrial tissues, immunostained with antibodies directed against β-galactosidase (GLB1), H2AX, HMGB1, p16ink4a and p21. All biopsies were taken 7 days after ovulation.

The performed Spearman analysis revealed significant positive correlation only between the percentage of p21-stained cells in endometrial stroma and glands and patient's age (R=0.53; p=0.002 and R=0.54; p=0.001, respectively) (**Fig. 2**). On the contrary, the percentage of immunostained cells against the other four IHC markers did not show significant correlation with age (**Table 2**).

In agreement with the impact of p21 in orchestrating replicative senescence, which has been already well known (Mirzayans *et al.,* 2012), we found a close association between this biomarker and patients age. Many age-related events activate tumour suppressor pathways, governed by p53 and pRB, transcriptional regulators that establish and maintain the senescence arrest (Herbig *et al.,* 2006; Campisi and d'Adda di Fagagna, 2007). Telomere shortening mechanism is tightly bound with the age related senescence, which is connected to activation of cyclin-dependent kinase inhibitor p21 (Choudhury *et al.,* 2007).

On the basis of the obtained results, it could be suggested that H2AX, HMGB1 and $p16^{ink4a}$ may be more valuable for detection of stress-induced senescence than age-related senescence in human endometrium. These markers could be permanently or irregularly induced by stress stimuli, which cause accumulation of double-strand DNA breaks in the process of stress-induced premature senescence. Unlike replicative senescence, this type is independent of telomere length or function (Zhang, 2007; Sikora *et al.,* 2011). Cyclin-dependent kinase (CDK) inhibitor, $p16^{INK4A}$, is usually discussed in relation to the stress-induced senescence (Robles and Adami, 1998). However, its role in the process of senescence is still not fully understood. Recently it was shown that p16 could even negatively or positively regulate apoptotic cell death depending on the stimuli (Al-Mohanna *et al.,* 2004; Hu *et al.,* 2011). DNA damage response proteins such as H2AX are also usually stress activated and act upstream of p53 and are required for the senescence-associated secretory phenotype (Rodier *et al.,* 2009). In addition, HMGBI expression is influenced by stress factors and it has a supplementary function as a secreted protein with a central role in inflammation caused by cell or tissue damage (Bianchi, 2007; Yamada and Maruyama, 2007).

Table 2. Correlation coefficient (R) between women age and the percentage of immunoreactive cells for the studied five senescence biomarkers (β-galactosidase, H2AX, HMGB1, $p16^{ink4a}$ and p21) in endometrial stroma and glands.

Senescence Biomarker	Endometrial stroma		Endometrial glands	
	R coefficient	p-Value	R coefficient	p-Value
β-galactosidase	0.27	NS	0.21	NS
H2AX	0.17	NS	0.11	NS
HMGB1	0.06	NS	0.15	NS
$p16^{ink4a}$	0.22	NS	0.08	NS
p21	0.53	0.002	0.54	0.001

***NS – not significant (p>0.05)**

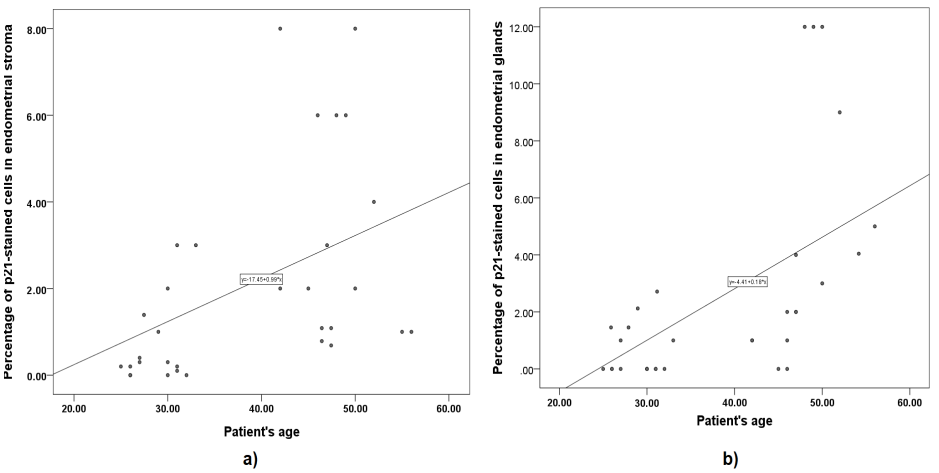

Fig. 2 Scatter plot with line of best fit showing a positive correlation between the patient's age and the percentage of p21-stained cells in (a) human endometrial stroma and (b) glands (n=45).

CONCLUSION

The most appropriate and easy detectable immunohistochemical biomarker for cellular age-associated senescence in human endometrium among the studied five markers for senescent cells (β-galactosidase, H2AX, HMGB1, $p16^{ink4a}$ and p21) is p21.

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DECLARATION OF INTEREST STATEMENT

All authors declare no conflict of interest.

AUTHORS CONTRIBUTION STATEMENT

D.P., G.S. and T.C. conceived the experiment; D.P., N.V., and T.C. conducted the experiment; D.P., T.C., G.S and R.G. analysed the results. D.P. and R.G. wrote the main manuscript text and prepared tables and figures. T.C., G.S. and N.V edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

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