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ORIGINAL ARTICLE

Evaluation of serum M30 and M65 activity in patients with stage-I endometrial cancer

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ABSTRACT

We aimed to analyse the prognostic value of serum oxidative stress parameters and apoptotic markers of serum M30/65 levels in endometrial cancer patients. Serum M30/65 levels and oxidative stress parameters were evaluated in 52 women with stage I endometrial cancer ($n = 26$) and a control group of healthy females ($n = 26$). The total antioxidant status ($p = .002$), oxidative stress index ($p = .003$) and serum M30/65 levels ($p < .001$) were significantly higher in women with stage-I endometrial cancer in comparison to the control group. Furthermore, serum M30/65 levels were significantly lower on post-operative day 8, compared to preoperative levels ($p = .001$ and $p < .001$, respectively), in the endometrial cancer group. Although impaired apoptotic activity plays a crucial role in the aetiopathogenesis of endometrial cancer, oxidative stress may be instrumental in malignant transformation. We concluded that measurement of M30/65 levels would be beneficial in the follow-up of women with endometrial cancer.

IMPACT STATEMENT

- **What is already known on this subject:** Although M30 has been evaluated as a marker of apoptosis in tissue samples from women with endometrial cancer **(**EC), no previous studies have simultaneously analysed serum M30 and M65 levels and oxidative stress in patients with stage-I EC.
- **What the results of this study add:** Total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI) and serum M30/65 levels were significantly higher in women with stage I EC in comparison to the control group. Furthermore, serum M30/65 levels were significantly lower on postoperative day 8, compared to preoperative levels, in the EC group. The fact that pre-operative M30/M65 levels were higher than the post-operative levels may be very important in early-stage EC
- **What the implications are of these findings for clinical practice and/or further research:** Although impaired apoptotic activity plays a crucial role in the aetiopathogenesis of EC, oxidative stress may be instrumental in malignant transformation. The fact that serum M30/M65 levels decreased in accordance with the reduction of post-operative tumour burden led us to conclude that measurement of M30/65 levels would be beneficial in the follow-up of women with EC.

KEYWORDS

Endometrial cancer; apoptosis; oxidative stress; serum M30 level; serum M65 level

Introduction

Endometrial cancer (EC) is the most frequently observed type of gynaecological cancer in developed countries, and it annually affects 15–20 women per 100,000. Approximately 75% of women are diagnosed at an early stage, and the 5-year survival rate is 83% (Sorosky 2008; Boll et al. 2013).

Apoptosis is a programmed cell death process that contributes to the homoeostasis of multicellular organisms. It is a process that helps the organism to annihilate damaged, dislocated or unnecessary cells (Carlson and Mutter 2008). Any failure in the exposure of these cells to appropriate apoptotic cell death may lead to the formation of numerous diseases, such as viral infections and cancer (Jemal et al. 2011). Recent studies have shown that serum M30/M65 levels are correlated with apoptotic activity and could be used as biomarkers in gestational trophoblastic neoplasia, ovary, stomach and kidney cancers (Ellis et al. 1991; Bilici et al. 2011; Yildiz et al. 2013a; Incebiyik et al. 2016a, 2016b). M30 is a caspase-cleaved pattern of cytokeratin-18 (CK-18) that is liberated from the extracellular area secondary to cell membrane damage all along the apoptosis process. M65 explicits all fragments of CK-18 that are discharged into the circulation all the while both tissue necrosis and apoptosis (Thompson 1995). The levels of M30 and M65 in the circulation can be evaluated using the enzyme-linked immunosorbent assay (ELISA) method (Ustaalioglu et al. 2013; Yildiz et al. 2013b).

Although M30 has been evaluated as a marker of apoptosis in tissue samples from women with EC (Wu et al. 2003), no previous studies have simultaneously analysed serum M30 and M65 levels and oxidative stress in patients with stage I

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EC. Therefore, the present study analysed whether serum M30 and M65 levels and oxidative stress parameters were different in patients with stage-I EC compared to in a control group.

Materials and methods

This prospective multicentre study was conducted in the Departments of Obstetrics and Gynaecology, Mugla Sitki Kocman University, Mugla, Turkey and Clinical Biochemistry, Harran University, Sanliurfa, Turkey, between October 2017 and June 2018. Serum M30/65 levels and oxidative stress parameters were evaluated in 52 women with stage I EC ($n = 26$) and a control group of healthy females ($n = 26$). Preoperatively, all patients are evaluated with medical history, physical-vaginal-pelvic examination, chest X-ray, transvaginal ultrasound and abdominal MRI, papanicolau smear. The EC was histopathologically diagnosed, by endometrial sampling and the surgical staging scheme of the International Federation of Gynaecology and Obstetrics (FIGO) was used for staging in frozen section examinations. All frozen sections were evaluated by the same pathologist that experienced in gynaecologic oncology. All surgical procedures performed by the same surgical team that experienced in gynaecologic oncology in Mugla Sitki Kocman University Training and Research Hospital. Totally, 61 patients with stage I EC were operated during the study period, but nine of them were excluded from the study due to not meeting the required criteria. Abdominal inspection, peritoneal washing, total laparoscopic hysterectomy and bilateral salpingo-oopherectomy were carried out in all participants. Lymphadenectomy was not performed in all grade-1 participants with a myometrial involvement depth of less than 50%. Vaginal brachytherapy alone was carried out in the participants with stage IA grade 3 ($n = 2$) and stage IB grade 1 and 2 ($n = 4$ and $n = 2$) disease, and vaginal cuff brachytherapy and/or pelvic radiotherapy was performed in those participants with stage IB grade 3 ($n = 3$) disease. All participants were evaluated in terms of age, gravidity, parity, body mass index, menopausal status and FIGO stage and grade. The study procedure was described to all participants and their written consent was obtained. The study protocol conformed to the principles of the Declaration of Helsinki, and was acclaimed by the Medical Ethics Committee of Mugla Sitki Kocman University.

We adjusted the sample width conforming to the results obtained from the first 16 participants that we evaluated. From the differences, domineering a two-tailed α value of 0.05 (sensitivity 95%) and a β value of 0.20 (study power: 80%, effect size: 0.75), we ruled that at least 46 women were mandatory for a analysis comparing two groups (G-Power 3 power analysis programme) (Faul et al. 2007). Therefore, we agreed to include minimum 23 women in the groups.

Exclusion criteria

Exclusion criteria included pulmonary disease ($n = 2$), pulmonary hypertension ($n = 1$), inadequate cardiac function ($n = 3$), renal and hepatic dysfunction ($n = 2$) and coexisting or preceding malignant disease ($n = 1$). Women taking antioxidant vitamins (vitamin A, C and E) were also excluded, as these vitamins could affect oxidative stress level and apoptotic status.

Blood samples were collected from all patients to measure total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI) and M30 and M65 levels. Additionally, another series of blood samples was obtained from the women with EC on post-operative day 8 to analyse the effects of surgical intervention on serum M30 and M65 levels.

Blood samples

All blood samples were taken from the antecubital vein on an empty stomach at night and in the morning. The samples were collected in polypropylene tubes and centrifuged at 3000 *g* for 10 min. The obtained supernatant was then stored at −80 °C until the analysis.

Measurement of total antioxidant status

The TAS measurement was done with spectrophotometry using a commercial kit (Rel®; Assay Diagnostics). The conclusions were asserted as mmol trolox equivalent/L.

Total oxidant status

The TOS measurement was done with spectrophotometry using a commercial kit (Rel®; Assay Diagnostics). The results were declared as micromolar hydrogen peroxide per litre (μmol H_2O_2 equivalent/L).

Oxidative stress index

OSI was described as the percentage ratio of TOS to TAS levels. The findings were expressed as mmol trolox/L.

Serum M30 level

The serum M30 level was calculated with ELISA using PEVIVA M30 Apoptonse kits (Prod. No.10010). The results were given as U/L.

Serum M65 level

The serum M65 level was calculated with ELISA using PEVIVA M30 Apoptonse kits (Prod. No.10020). The results were declared as U/L.

Statistical analysis

The statistical analysis was performed via Statistical Package for the Social Sciences software, version 16.0 (SPSS for Windows Chicago, IL) programme. All data were indicated as mean and standard deviation values. The Kolmogorov–Smirnov calculation test was used to analyse

the distribution of the data, the independent samples *t*-test was used for the between-groups comparison and the chi-square test was used to evaluate non-numerical data. The paired-samples *t*-test was used to analyse the pre- and post-operative laboratory variables, and a receiver-operating curve (ROC) analysis was constructed to evaluate diagnostic performance and optimal cut-off values for M30 and M65 in the EC group. $p < .05$ was confirmed as being statistically significant.

Results

The demographical data of all the women incorporated in the study are shown in Table 1. All participants had the endometrioid adenocarcinoma histological type. A total of 17 of the participants had stage IA EC, while the remaining nine participants were had stage IB disease. Of the 17 participants with stage IA EC, 11 were determined as having grade-1 disease, four patients as having grade 2 and the remaining two patients as having grade-3 disease. Of the nine patients with stage IB EC, four were determined as having grade-1 disease, two patients as having grade 2 and three patients as having grade-3 disease.

Serum M30/M65, TAS, TOS and OSI levels for both groups are shown in Table 2. While the TOS and OSI values, parameters of oxidative stress, were significantly higher in women with EC in comparison to the control group, the TAS level, a marker of the antioxidant status, was lower (Table 2). In the evaluation made between the stages and oxidative stress parameters in women with EC, while the TOS and OSI levels were lower in those with stage IA disease, compared to those with stage IB disease, the TAS level was higher. However, it was observed that the difference was significant only in terms of the TAS levels ($p = .163$, $p = .254$ and $p = .023$, respectively). In addition, a statically significant difference between the histological grade and the oxidative stress parameters (TAS, TOS, OSI) was not found ($p = .970$, $p = .943$ and $p = .917$, respectively).

Table 1. Demographic characteristics in the patients with endometrial cancer and control groups.

	EC	Control	*p*
Age, years	58.07 ± 3.2	56.96 ± 6.4	.433
Gravidity, *n*	2.9 ± 1.6	3.1 ± 1.2	.637
Parity, *n*	1.6 ± 1.3	2.1 ± 1.1	.228
BMI, kg/m^2	28.94 ± 3.4	30.2 ± 2.8	.158
Menopause, *n*	18	22	.188

BMI: body mass index; EC: endometrial cancer.
Data are defined as means and standard deviations.

Table 2. TAS, TOS, OSI, M30 and M65 levels in the patients with the endometrial cancer and control groups.

	EC	Control	*p*
TAS (mmol trolox equivalent/L)	0.88 ± 0.1	1.01 ± 0.19	.005[a]
TOS (μmol H_2O_2 equivalent/L)	34.56 ± 7.71	28.83 ± 4.1	.002[a]
OSI (mmol trolox)	3.31 ± 0.88	2.69 ± 0.45	.003[a]
M30 (U/L)	169.65 ± 42.30	119.03 ± 27.35	<.001[a]
M65 (U/L)	341.76 ± 77.10	227.65 ± 41.72	<.001[a]

OSI: oxidative stress index; SD: standard deviation; TAS: total antioxidant status; TOS: total oxidant status.
[a]Independent samples t-test.

The comparison of serum M30/M65 levels by stage in women with EC is presented in Table 3. Their serum M30/M65 levels, considered as the markers of apoptotic and necrotic activities, were higher than those of the control group ($p < .001$, $p < .001$, respectively) (Table 2). The independent samples *t*-test was used to determine whether there was a relationship between the stage of cancer and the serum M30 and M65 levels in the women with EC, and the results showed that these levels were higher in women with stage IB disease; however, the difference not statistically significant (M30; 167.05 ± 47.20, 174.55 ± 35.08, $p = .676$; M65; 320.59 ± 66.52, 381.78 ± 83.51, $p = .052$).

The result of the ROC analysis with regard to serum M30/M65 levels in women with EC is presented in Figure 1, and the area under the curve (AUC) was determined as 0.864 for M30. When the cut-off value for M30 was 123 U/L, the sensitivity was determined as 84.6% and the specificity as 69.2%. The AUC was determined as 0.930 for M65. When the cut-off value for M65 was as 266 U/L, the sensitivity was determined as 88.5% and the specificity as 76.9%.

It was observed that serum M30 and M65 levels had reduced significantly on the eight day of postoperative period compared to the preoperative period ($p = .001$ and $p < .001$, respectively), as shown in Table 4.

Discussion

This study analysed whether there was a discrepancy between women with early-stage EC and healthy controls in

Table 3. The comparison of serum M30/M65 levels by stage in women with EC.

	Stage-IA EC	stage-IB EC	*p*
M30 U/L	167.05 ± 47.20	174.55 ± 35.08	.676[a]
M65 U/L	320.59 ± 66.52	381.78 ± 83.51	.052[a]

EC: endometrial cancer; SD: standard deviation.
[a]Independent samples *t*-test.

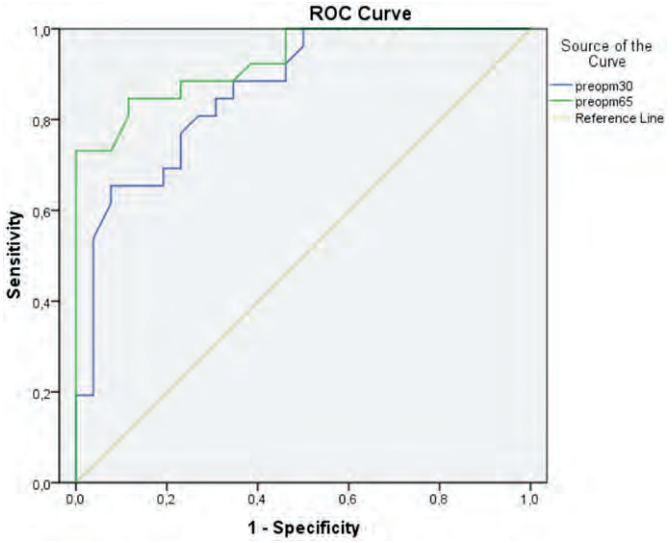

Figure 1. Area under the curve according to receiver-operating curve analysis for serum M30 and M65 levels.

Table 4. Comparison of patients with EC corcerning preoperative and postoperative serum M30 and M65 levels.

	Pre-operative	Post-operative	*p*
M30 U/L	169.65 ± 42.3	153.73 ± 41.7	.001
M65 U/L	341.76 ± 77.1	316.34 ± 77.6	<.001

Data are defined as means and standard deviations.
EC: endometrial cancer.

terms of serum oxidative stress markers and M30 and M65 levels. The findings can be summarised as follows: (a) in the women with stage-I EC, the TOS and OSI levels, the serum oxidative stress markers, were significantly higher compared with those of the healthy controls, while the TAS levels, the serum antioxidant markers, were significantly lower; (b) serum M30 and M65 levels were significantly higher in the women with early stage EC compared to the control group; (c) the serum M30/M65 levels were significantly lower in accordance with the reduction of tumour burden in the postoperative period.

The relationship between impaired antioxidant activity and cancer development has been known exactly. Severe oxidative stress can play a significant role in carcinogenesis, leading to damage and mutation of tumour suppressor genes. Studies performed on tissue and erythrocytes obtained from women with EC showed that antioxidant enzyme activity significantly decreased, while lipid peroxidation increased considerably. In our previously published study, we found that antioxidant activity decreased in contrast with the increasing levels of oxidative stress markers in women with early stage EC (Arioz et al. 2009). In the present study, the TAC level was lower, while the TOS and OSI levels were higher in the women with stage 1 EC compared to the controls. This led us to conclude that oxidative stress could play a role in the development of EC. Nevertheless, the findings are not sufficient to allow us to comment with regard to whether the increased oxidative stress was the cause or effect of EC aetiopathogenesis.

Apoptosis plays an essential role in the physiology of the menstrual cycle. In addition, increased cellular apoptosis is frequently observed in the progression from endometrial hyperplasia to adenocarcinoma through atypia. Moreover, it has been asserted that increased apoptosis could be an early morphological indicator of constant abnormal endometrial growth (Arends 1999; Stewart et al. 1999). Abnormal changes found in pro- or anti- apoptotic proteins can lead to EC development. For example, excessive production of the anti-apoptotic Bcl-2 protein may block apoptosis and extend cell survival time. As a result, carcinogenesis or malignant progression may occur (Giatromanolaki et al. 1998; Morsi et al. 2000; Saitoh et al. 1999).

Apoptosis is described as the energy-dependent programmed cell demise that occurs as a result of physiological and pathological events (Incebiyik et al. 2016a), and two pathways are involved in its induction: the extrinsic pathway, which is a death receptor-initiated, caspase-8-mediated pathway, and the intrinsic pathway, which is a mitochondria-initiated, caspase-9-mediated pathway (Chan et al. 2009). M30/M65 antibodies that show the caspase-cleaved activation products of CK-18 in circulation can be used both in the morphological assessment of tissue samples and in the determination of apoptotic activity, via the use of electron and light microscopes (Chiu et al. 2001; Sharp et al. 2010).

CK-18 is a representative of the intermediate filament group, which is the major building block of the cytoskeleton, and it is frequently observed in endothelial and epithelial cell strains (Bilici et al. 2011). The intact and caspase-cleaved forms of CK-18 are markers for the demise of cancer cells, and are discharged into circulation while both apoptotic and non-apoptotic cell death (Oven Ustaalioglu et al. 2012; Yildiz et al. 2013a). CK-18 fragments can be determined in the circulation via ELISA, using M30 and M65 antibodies (Chiu et al. 2001). M30, a monoclonal antibody, identifies only the caspase-cleaved fragments of CK-18, but it cannot determine natural and intact CK-18. In addition, M30 cannot determine viable and necrotic cells, but it can show apoptotic cells (Ueno et al. 2005). In contrast, M65 can determine all CK-18 fragments released into the circulation during both apoptosis and necrotic cell death (Ustaalioglu et al. 2013; Yildiz et al. 2013a). Some studies have revealed that M30 and M65 levels are significant markers of tumour burden in ovary, stomach, colorectal, kidney, lung and head and neck cancers (Ozturk et al. 2009; Bilici et al. 2011; Oven Ustaalioglu et al. 2012; Ustaalioglu et al. 2013; Yildiz et al. 2013a; Incebiyik et al. 2016a, 2016b). In addition, it was found that M30 activity increased in women with EC in a study performed on paraffin-embedded samples (Morsi et al. 2000). Moreover, Wu et al. showed an important correlation between the M30 level in EC and the biological behaviour of the tumour and the prognosis (Wu et al. 2003). In the present study, serum M30/M65 levels were higher in the women with EC compared to the control group. This is critical, as it shows that apoptosis might play a crucial role in the aetiopathogenesis of EC. In addition, these findings emphasise the fact that the M30 and M65 levels can be markers of EC. Moreover, pre- and post-operative findings showed that M30 and M65 levels could be helpful in the assessing the progression of the women with EC and the follow-up of their tumour burdens.

Although it is difficult to generalise the findings of this study, due to its single-centre design, small sampling size and short follow-up time, the fact that pre-operative M30/M65 levels were higher than the post-operative levels may be very important in early stage EC. However, more comprehensive studies and long-term data are required to verify our findings.

Conclusion

In the present study, serum M30/M65 levels and oxidative stress markers were higher in the women with EC compared to the control group. Therefore, while oxidative stress is instrumental in malignant transformation, impaired apoptotic status can play a role in EC development. Reduction in M30/M65 levels in accordance with the reduction in the post-operative tumour burden emphasises that M30/M65 measurements could be used in the follow-up of women with EC. Nevertheless, the findings of the present study must be verified in more extensive studies using larger sample sizes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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