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ORIGINAL PAPER

Overexpression and ratio disruption of ΔNp63 and TAp63 isoform equilibrium in endometrial adenocarcinoma: correlation with obesity, menopause, and grade I/II tumors

Eleni Vakonaki · Nikolaos Soulitzis · Stavros Sifakis · Danae Papadogianni · Dimitrios Koutroulakis · Demetrios A. Spandidos

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Abstract
Purpose p63 plays an important role in several intracellular processes such as transcription activation and apoptosis. p63 has two N-terminal isoforms, TAp63 and ΔNp63. TAp63 isoform has p53-like functions, while ΔNp63 acts as a dominant negative inhibitor of the p53 family and is considered oncogenic. Although p63 and its isoforms are overexpressed in a wide variety of human malignancies such as cervical, head and neck, and lung cancer, their role in endometrial carcinoma has not been investigated.
Methods We measured by quantitative real-time polymerase chain reaction the mRNA expression of TAp63 and ΔNp63 in a series of 20 endometrioid adenocarcinomas paired with adjacent normal tissue.
Results TAp63 isoform exhibited 1.8-fold overexpression in malignant samples, while ΔNp63 was 4.3-fold overexpressed in cancer specimens. Further analysis revealed that the ΔN/TA isoform ratio shifted from 0.5 in normal samples to 1.2 in tumor specimens. Statistical analysis also revealed an association of TAp63 expression with high body mass index ($p = 0.034$), late menopause ($p = 0.020$), and lower tumor grade ($p = 0.034$). ΔNp63 was also correlated with grade I/II tumors ($p = 0.044$).
Conclusions These results indicate that both p63 isoforms and especially ΔNp63 play an important role in the development and progression of grade I/II endometrial adenocarcinoma, especially in obese and late-menopause women.

Keywords p63 · Real-time PCR · mRNA · Endometrium · Biomarkers

Eleni Vakonaki and Nikolaos Soulitzis have contributed equally to this work.

E. Vakonaki · N. Soulitzis · D. Papadogianni · D. A. Spandidos (✉)
Laboratory of Clinical Virology, Medical School, University of Crete, P.O. Box 2208, Heraklion 71003, Crete, Greece
e-mail: spandidos@spandidos.gr

S. Sifakis · D. Koutroulakis
Department of Obstetrics and Gynecology, University Hospital of Heraklion, Heraklion, Crete, Greece

Introduction

Endometrial carcinoma is the fourth most common malignancy among women after breast, lung, and intestine cancer (Siegel et al. 2011), with endometrioid adenocarcinoma being the most frequent histological subtype. The risk of developing endometrial cancer depends on factors such as age, estrogen therapy, the presence of metabolic syndromes like diabetes, and estrogen levels after menopause. There are two types of the disease, the first a high risk carcinoma, correlated with overexpression of PTEN, hTERT, ErbB2, c-myc, and p53, while the second type is a low risk malignancy, correlated with microsatellite instability (MI) and mutations in PTEN, K-Ras, and b-catenin genes (Kapucuoglu et al. 2007). The histological grade of endometrial cancer is associated with tumor staging, prognosis, lymph-node metastasis, and myometrial invasion, parameters that also determine the selection of treatment. The excellent prognosis of early-stage endometrial cancer renders it one of the most curable malignancies, with high 5-year survival rates.

The p53 tumor suppressor gene plays an important role in human malignancies, including endometrial cancer. p63 gene is an homolog of p53, is located on 3q27-29, and contains 15 exons (Levrero et al. 2000). These genes

encode for two proteins with three domains in common: a transactivation domain, a DNA binding domain, and an oligomerization domain. The transactivating isoforms TAp63 (containing TA domain) are generated by a promoter upstream of exon 1 (P1), and the ΔNp63 (lacking TA domain) isoforms are generated by an alternative promoter located in intron 3–4 (P2) (Fig. 1). TAp63 isoform is the full-length protein and, like p53, induces cell-cycle arrest and apoptosis. ΔNp63 has an oncogenic role in cancer progression and acts as a dominant negative inhibitor of the p53 family (Wu et al. 2003). Studies in p63 knock-out mice did not reveal tumors, indicating a limited role of p63 in murine oncogenesis (Melino et al. 2003), even though other types of alterations have been observed, including skin and other stratified epithelial dysplasias, limb truncation, and craniofacial abnormalities, probably due to an alteration of the apical ectodermal ridge. In humans, p63 overexpression has been observed in various types of malignancies, such as breast, pancreatic, and uterine cervical cancer, as well as in a number of syndromes known as ectodermal dysplasias (Graziano and De Laurenzi 2011).

p63 overexpression is responsible for increased cell proliferation, by altering cell response to growth arrest signals, probably by inhibiting the cyclin kinase inhibitors (CKIs) p21 and p57. The two p63 isoforms, ΔN and TA, seem to have distinct roles on the cell cycle; ΔNp63 is clearly an oncogenic agent, protecting cells from cell death, through a dominant negative effect on TAp63/p53/TAp53 expression. It can also activate the β-catenin signaling pathway, contributing further to oncogenesis (Patturajan et al. 2002). TAp63 on the other hand acts as a tumor suppressor, through its ability to induce senescence, together with p21 and Rb, independently of the p53 expression status, as shown in studies on TAp63 knock-out mice demonstrating an increased number of primary and metastatic tumors.

In the present study, we measured the mRNA expression levels of TA and ΔN p63 isoforms in a series of 20 endometrial malignant samples paired with adjacent normal tissue and correlated the results with patients' clinicopathological characteristics.

Materials and methods

Study subjects

Endometrial tumors and adjacent normal tissue were collected from 20 women with endometrial cancer at the Department of Obstetrics and Gynecology, Heraklion University Hospital, Crete, Greece. Samples were obtained after total hysterectomy. Mean patient age at the time of surgery was 60.7 years (range: 43–83). Tissues were divided into two; half was sent for histopathological examination (which determined that all tumor cases were endometrioid adenocarcinomas), and the other half (approximately 40 mg) was snap frozen and stored at −80 °C until used in our analysis. The clinicopathological characteristics of our patients are shown in Table 1. The study was approved by the Ethics Committee of the University of Crete, and written informed consent was obtained from all participants.

RNA extraction

Tissue samples (∼20 mg) were homogenized in TRI reagent (Molecular Research Center Inc., Cincinnati, OH) using a power homogenizer and incubated at room temperature, followed by the addition of chloroform and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75 % ethanol, and resuspended in 20–30 μl of DEPC-treated water. RNA concentration and purity were calculated after measuring on a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE) its 260 nm absorbance and 260/280 nm absorbance ratio, respectively.

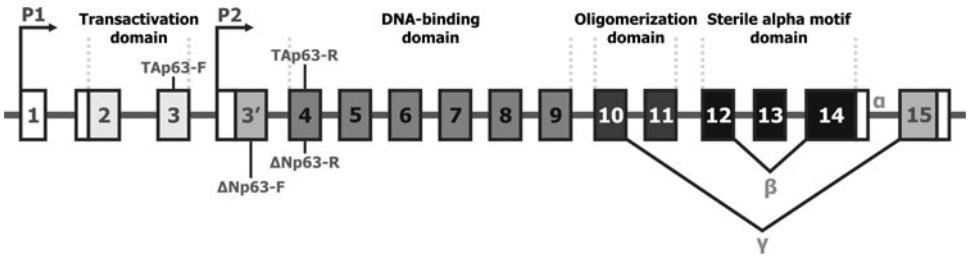

Fig. 1 p63 gene structure. TAp63 transcripts are encoded from the P1 promoter upstream of exon 1, while ΔNp63 transcripts are encoded from the alternative P2 promoter upstream of exon 3′. The various protein domains are also demonstrated (corresponding to different colored exons), along with the three common carboxyl-terminus isoforms (α, β, and γ). The location of the TAp63 primer pair in exons 3 (TAp63-F) and 4 (TAp63-R) and of the ΔNp63 primer set in exons 3′ (ΔNp63-F) and 4 (ΔNp63-R) are also displayed

Table 1 Patients' clinicopathological characteristics

Characteristic	No. of patients (%) ($n = 20$)
Age	
Mean ± SD, years	60.7 ± 10.1
Range	43–83
BMI	
Mean ± SD, Kg	31.6 ± 5.5
Parity	
1-2	8 (40.0 %)
3+	12 (60.0 %)
Abortions	
Yes	13 (65.0 %)
No	7 (35.0 %)
Menopause	
Mean ± SD, years	50.9 ± 4.5
Range	43–55
Smoking	
Yes	2 (10.0 %)
No	18 (90.0 %)
Diabetes	
Yes	9 (45.0 %)
No	11 (55.0 %)
Hypertension	
Yes	13 (65.0 %)
No	7 (35.0 %)
Hyperlipidemia, hypercholesterolemia	
Yes	9 (45.0 %)
No	11 (55.0 %)
Thyroid	
Yes	10 (50.0 %)
No	10 (50.0 %)
Family Ca history	
Yes	9 (45.0 %)
No	11 (55.0 %)
Previous Ca	
Yes	5 (25.0 %)
No	15 (75.0 %)
Tumor grade	
I	8 (40.0 %)
II	6 (30.0 %)
III	6 (30.0 %)
Tumor stage (FIGO 2008)	
IA	16 (80.0 %)
IB	4 (20.0 %)
Endometriosis	
Yes	7 (35.0 %)
No	13 (65.0 %)
Endometrial hyperplasia	
Yes	12 (60.0 %)
No	8 (40.0 %)

cDNA preparation

RNA was retrotranscribed utilizing the PrimeScript 1st strand cDNA Synthesis kit (Takara Bio Inc., Japan). For 5 min at 65 °C, 5 μM of random 6mers, 4 μM dNTPs, 1 μg template RNA, and water up to 10 μl were incubated. To the reaction mixture, 5 μM of template RNA Primer mixture, 20U of RNase inhibitor, 5× PrimeScript™ Buffer, and 200U of PrimeScript™ RTase were added (total volume 20 μl) and incubated for 10 min at 30 °C for the annealing of random hexamers, and for 30 min at 42 °C for the elongation of cDNA targets. The enzyme was inactivated by incubation at 95 °C for 5 min followed by cooling on ice. cDNA aliquots were stored at −20 °C until used.

Quantitative real-time PCR (qRT-PCR)

For real-time PCR reactions, the KAPA SYBR FAST qPCR kit (Kapa Biosystems Inc., Woburn, MA) was used. cDNA (1 μl) was mixed with 1× Kapa Master Mix, 0.4 μl of Rox reference dye, and 30 nM of TAp63 and ΔNp63 primer pairs, respectively. Primers were designed to span a least one intron to avoid amplification of contaminating genomic DNA (Table 2; Fig. 1). PCR conditions for TAp63 and ΔNp63 were 3 min at 95 °C, 40 cycles of 3 s at 95 °C, 25 s at 62 °C, 1 s at 72 °C, followed by melt-curve analysis. β-actin was used as internal control to normalize TAp63 and ΔNp63 expression levels. A representative pool of all samples was diluted in a series of six 2× dilutions, which were used to construct a standard curve for the quantification process. PCR reactions were carried out on a Mx3000P Real-Time PCR Thermocycler (Agilent Technologies Inc., Santa Clara, CA), using MxPro software (version 4.1). The normalized expression of each target gene was calculated using the following formula:

$$\text{Normalized sample} = (1 + E_{p63})^{-\Delta Ctp63} / (1 + E_{\beta\text{-actin}})^{-\Delta Ct\beta\text{-actin}}$$

Each value from tumor samples was divided by the value of the corresponding normal sample. Two-fold increased or decreased expression was considered overexpression or downregulation, respectively. For verification, all PCR products were analyzed on 2.5 % (w/v) agarose gels, stained with ethidium bromide, and photographed on a AlphaImager™ (ProteinSimple Inc., Santa Clara, CA) UV transilluminator.

p63 P1 and P2 promoters CpG islands search

The first step in checking the methylation status of a promoter region is to identify its CpG islands. p63 nucleotide sequence was obtained from Ensembl (http://www.ensembl.org/). Using the MethPrimer (http://www.urogene.org/

methprimer/) and EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) software applications, we searched the two p63 promoters (4 Kb upstream and 1 Kb downstream of the corresponding exons 1 and 3′, respectively), for CpG islands, in order to design primer sets for the methylation analysis.

Statistical analysis

TAp63 and ΔNp63 mRNA levels were first evaluated by the one-sample Kolmogorov–Smirnov goodness of fit test, in order to determine whether they followed a normal distribution pattern. Depending on the results, Pearson's correlation or the non-parametric Spearman rank correlation was used to examine their relation pair-wise and their association with continuous variables (age, BMI, etc.). Moreover, their association with categorical data (tumor stage and grade, etc.) was examined using Student's t test (after examining for equality of variances with Levene's test), or its non-parametric equivalents Mann–Whitney U and Kruskal–Wallis H tests. Finally, the chi-square (χ^2) test, using Fisher's exact test when indicated by the analysis, was used to examine TAp63 and ΔNp63 expression status with the various clinicopathological parameters after stratification. All statistical analyses were 2-sided and performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95 % level (p value < 0.05).

Results

In the present study, the mRNA expression profile of p63 gene isoforms (TAp63 and ΔNp63) was examined using real-time PCR in 20 women who had undergone surgery for endometrial cancer.

TAp63 isoform was overexpressed in 4/20 (20 %) samples, downregulated in 3/20 (15 %), while in 3/20 (15 %) specimens, its expression was normal. Additionally, in 6/20 samples (30 %), TAp63 was expressed only in tumor specimens, while in 4/20 samples (20 %), TAp63 was not expressed in both normal and malignant endometrium (Table 3; Fig. 2).

ΔNp63 was overexpressed in 4/20 (20 %) and downregulated in 3/20 (15 %) specimens. In 6/20 samples (30 %), ΔNp63 was expressed only in tumor specimens, while in 7/20 samples (35 %), ΔNp63 was not expressed in both normal and malignant tumors (Table 3; Fig. 2).

Further analysis showed that TAp63 isoform exhibited a 1.8-fold overexpression in malignant samples (from 0.83 in normal samples to 1.45 in tumor specimens), while ΔNp63 was 4.3-fold overexpressed in cancer specimens (from 0.42 in normal samples to 1.81 in tumor specimens). Additionally, the ΔN/TA isoform ratio shifted 2.5-fold in favor of ΔNp63, from 0.50 in normal samples to 1.24 in tumor specimens. Interestingly, adding the expression levels of TAp63 and ΔNp63, the expression of total p63 increased 2.6-fold in malignant samples (from 1.24 in normal samples to 3.26 in tumor specimens) (Fig. 3).

Statistical analysis revealed that TAp63 isoform was overexpressed in obese women versus women with lower body mass index (1.62 ± 0.11 vs. 0.64 ± 0.20, $p = 0.034$). TAp63 expression was also higher in women with menopause at 50+ years old versus women with menopause at an earlier age (1.72 ± 0.08 vs. 0.67 ± 0.18, $p = 0.020$). Additionally, TAp63 mRNA levels decreased in Grade III tumors versus Grade I and II tumors (2.00 ± 0.44 vs. 0.77 ± 0.19, $p = 0.034$). Finally, ΔNp63 isoform expression was higher in Grade I/II tumors versus Grade III tumors (1.67 ± 0.32 vs. 0.58 ± 0.14, $p = 0.044$). Further analysis did not reveal any other statistically significant associations between expression status of p63 isoforms and patients' clinicopathological characteristics (Fig. 4).

Finally, in silico analysis of the two p63 promoters (P1 and P2) that encode the TA and ΔN isoforms revealed that both promoters lack CpG islands, meaning that the expression of p63 transcripts is not regulated by promoter methylation.

Discussion

p63 plays many diverse intracellular roles that depend on the expression pattern of its isoforms. In particular, TAp63

Table 2 Primer sequences, annealing temperatures and amplicons sizes of the study genes

Primer pair	Sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)
TAp63-F	AAG ATG GTG CGA CAA ACA AGA T	60	155
TAp63-R	GGG ACT GGT GGA CGA GGA		
ΔNp63-F	TGT ACC TGG AAA ACA ATG CCC A	60	103
ΔNp63-R	GAC GAG GAG CCG TTC TGA ATC T		
β-actin	CGG CAT CGT CAC CAA CTG	60	70
	GGC ACA CGC AGC TCA TTG		

seems to induce cell apoptosis, while ΔNp63 has an oncogenic activity.

In our study, the mRNA expression of TA and ΔN p63 isoforms was evaluated by real-time PCR, in 20 women with endometrial cancer. We found that the TAp63 isoform exhibited a 1.8-fold overexpression in malignant samples, while ΔNp63 was 4.3-fold overexpressed in cancer specimens (overall p63 upregulation: 2.6-fold). Statistical analysis revealed an association of TAp63 expression with menopause, obesity, and grade I/II tumors, a finding also observed for ΔNp63.

Koker et al. studied the expression of p63 in breast cancer, in which 86.7 % of patients with metaplastic carcinoma overexpressed p63. In contrast, in only 0.6 % of patients with non-metaplastic invasive carcinoma, such an overexpression was observed, while in phyllodes tumors and sarcomas, p63 was not expressed at all (Koker and Kleer 2004). According to the study of De Biase et al., p63 was overexpressed in 55.5 % of basal-like breast carcinoma, while such expression in luminal-type invasive breast carcinoma was lower, from 0.6 to 19.5 % (de Biase et al. 2010). TAp63 in particular was overexpressed in 75 % of samples. On the contrary, in a study conducted by Ribeiro-Silva et al., p63 overexpression was observed only in a small percentage of breast carcinoma samples (Ribeiro-Silva et al. 2003).

p63 was also upregulated in a variety of cancer types other than breast, such as cervical, salivary gland tumor, and lung cancer (Barbareschi et al. 2001). In lung cancer in particular, p63 was upregulated in several different histological subtypes, such as squamous cell carcinoma, large cell carcinoma, high grade neuroendocrine (NET) lung cancer, and sarcomatoid tumors, in which p63 overexpression was observed in ~90 % of samples (Lewis et al. 2005). On the contrary, Park et al. studied the expression level of p63 and its isoforms in bladder cancer and found that 53.2 % of samples exhibited reduced expression of TAp63 (Park et al. 2000).

ΔNp63 overexpression in our series of endometrioid adenocarcinomas is in agreement with a study conducted by Lin et al., in which 26.7 % of endometrial carcinomas overexpressed ΔNp63 (Lin et al. 2006). The fact that in 30 % of our samples ΔNp63 expression was tumor specific has also been observed before by Basturk et al., who studied

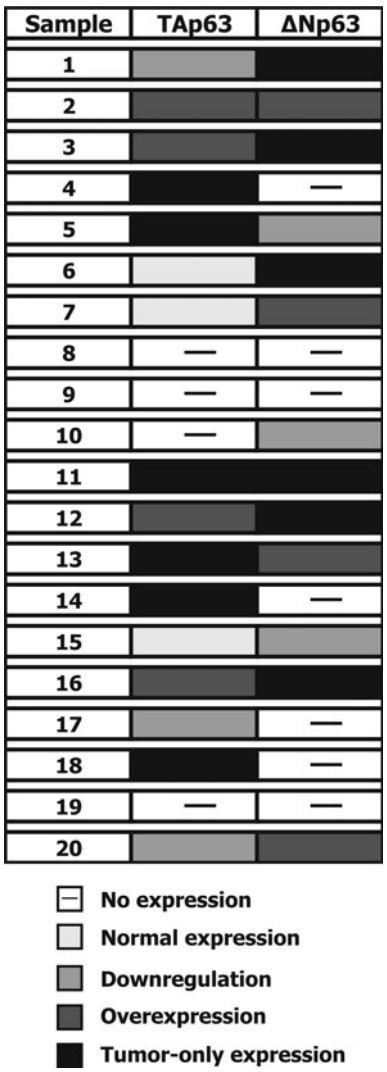

Fig. 2 Schematic representation of TAp63 and ΔNp63 expression profile in our series of endometrial carcinoma tissue samples

Table 3 Results of TAp63 and ΔNp63 expression analysis in normal and malignant endometrial tissue samples

	Overexpression (%)	Normal expression (%)	Reduced expression (%)	No expression (%)
TAp63				
Normal	–	10/20 (50.0)	–	10/20 (50.0)[a]
Tumor	10 (4 + 6)/20 (50.0)[a]	3/20 (15.0)	3/20 (15.0)	4/20 (20.0)
ΔNp63				
Normal	–	7/20 (35.0)	–	13/20 (65.0)[b]
Tumor	10 (4 + 6)/20 (50.0)[b]	–	3/20(15.0)	7/20 (35.0)

[a] In 6 samples, TAp63 was expressed only in tumor and not in the adjacent normal tissue

[b] In 6 samples, ΔNp63 was expressed only in tumor and not in the adjacent normal tissue

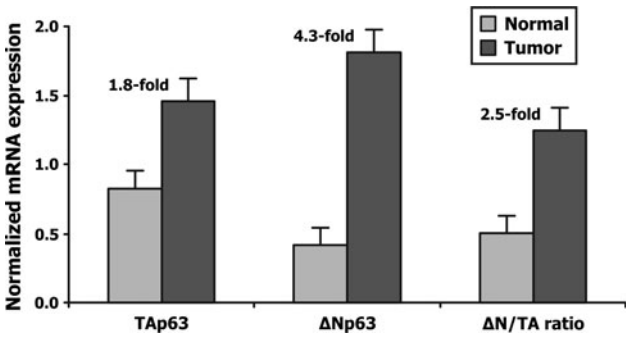

Fig. 3 *Bar* chart depicting TAp63 and ΔNp63 normalized expression, as well as ΔN/TA expression ratio in normal and malignant specimens, respectively. *Floating numbers* represent fold change between the two sample groups

the expression levels of ΔNp63 in normal pancreas and pancreatic neoplasia. Even though no ΔNp63 expression was found in normal pancreatic ducts, all squamous/transitional metaplasia samples showed strong and uniform nuclear positivity for this marker (Basturk et al. 2005). p63, and especially the ΔN isoform, was also strongly expressed in nuclei of squamous esophageal cancer cells, with expression decreasing as the distance from the tumor area increases (Cao et al. 2009).

ΔNp63 overexpression, even though having been observed in endometrial cancer only once before, is fairly common among other neoplasias and is usually correlated with advanced disease and poor survival/prognosis (Geddert et al. 2003; Marchini et al. 2008; Matsubara et al.

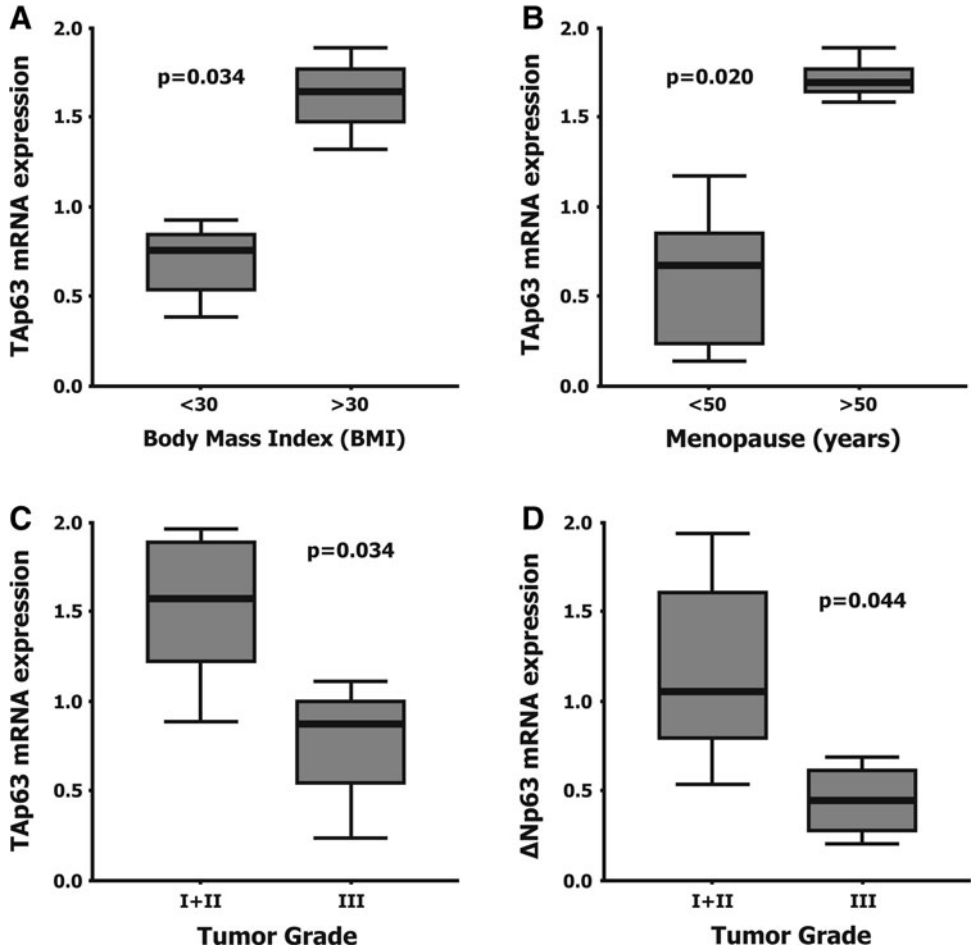

Fig. 4 *Box* and *whisker* plots depicting statistically significant associations of p63 isoforms with patients' various clinicopathological characteristics. **a** TAp63 isoform is overexpressed in obese women versus women with lower body mass index (1.62 ± 0.11 vs. 0.64 ± 0.20, $p = 0.034$). **b** TAp63 expression is higher in women with menopause at 50+ years old versus women with menopause at earlier age (1.72 ± 0.08 vs. 0.67 ± 0.18, $p = 0.020$). **c** TAp63 mRNA levels decrease in Grade III tumors versus Grade I/II tumors (2.00 ± 0.44 vs. 0.77 ± 0.19, $p = 0.034$). **d** TAp63 isoform expression is higher in Grade I/II tumors versus Grade III tumors (1.67 ± 0.32 vs. 0.58 ± 0.14, $p = 0.044$). All values are presented as mean ± standard deviation. The *thick line* near the center of each rectangular box represents the median value, the *bottom* and *top* edges of the box indicate the 1st (Q_1) and 3rd (Q_3) quartiles, and the ends of the *whiskers* depict the 10th (P_{10}) and 90th (P_{90}) percentiles. Statistical analysis was conducted with 2-tailed Mann–Whitney *U* test

2011). While the mechanism that activates this p63 isoform is not the same as the hypomethylation mechanism that activates the homolog ΔN isoforms of p73 (Daskalos et al. 2011), leading also to ΔN/TA p73 ratio disruption (Arvanitis et al. 2004), recent studies suggest that both β-catenin (Ruptier et al. 2011) and miR-203 (Yuan et al. 2011) seem to play an active role in its transcription regulation.

Although menopause (Sivridis and Giatromanolaki 2011) and obesity (Schmandt et al. 2011) are known risk factors for the development of endometrial carcinoma, this is the first study correlating p63 expression (particularly TAp63) with these risk factors, not only in endometrial malignancies but in cancer in general. Perhaps it would be worthwhile to further study p63 in obesity and menopause, in association with other malignant or pathological conditions. On the contrary, while p63 overexpression has been mainly observed in high grade tumors, such as lymphomas (Rushing et al. 2008) and meningiomas (Pruneri et al. 2005), we observed increased expression of both p63 isoforms not in grade III endometrial adenocarcinomas but in grade I/II ones. This discrepancy can probably be attributed to the different molecular profile that endometrial tumors have from the above-mentioned malignancies, although further studies are required to elucidate this.

The fact that no CpG islands exist in the two p63 promoters (P1 and P2) leads to the conclusion that p63 isoform upregulation or tumor-only expression cannot be attributed to decreased methylation of its promoters in tumor samples, since the gene is not methylated at all. Additionally, although p63 mutations are associated with at least six syndromes like EEC, AEC, Rapp–Hodgkin Syndrome, and Split Hand/Foot Malformation (Sifakis et al. 2001), there is no evidence that correlates p63 mutations with increased or decreased expression of p63 isoforms in cancer (Rinne et al. 2006). Therefore, overexpression of p63 isoforms depends on other epigenetics factors, such as phosphorylation (Deutsch et al. 2011; Kim et al. 2011), acetylation (Sasaki et al. 2008), or proteasome degradation (Maisse et al. 2003). Additionally, as Papagiannakopoulos et al. showed, miR-21 suppresses the expression of TAp63/p53, by creating a feedback loop between miR-21 and ΔNp63/p53/TAp63/p73 (Papagiannakopoulos et al. 2008). Moreover, by increasing the expression of mature miR-21, ΔNp63 can suppress the expression of tumor suppressor genes, including TAp63 and p53 (Boominathan 2010).

In conclusion, our study revealed that p63 isoforms are overexpressed in grade I/II endometrial adenocarcinoma in obese and late-menopause patients, and that in cancer samples, the ΔN/TA isoform ratio changes 2.5-fold in favor of ΔNp63. We believe that these results shed new light in the molecular profile of endometrial adenocarcinoma, which could lead to the establishment of TAp63 and ΔNp63 as new biomarkers for early detection and therapy of endometrial and other malignancies.

Conflict of interest The authors declare that they have no conflict of interest.

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