OSTEOPONTIN AND PTEN ISOFORMS EXPRESSION PATTERNS IN ENDOMETRIUM CARCINOMA CELL LINES

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INTRODUCTION

- Endometrial carcinoma (EC) is the most frequent gynecologic cancer in the US. In Brazil, it is on seventh position. It is classified in Type I and II (Table 1).
- Osteopontin (OPN) perform distinct roles in tumor progression, while the *Phosphatase and Tensin homolog* (PTEN) gene, act as a supressor gene. It has been reported that OPN expression is upregulated in tumors in which PTEN gene is mutated or deleted.
- OPN has three splicing isoforms (OPN-SI), named OPNa, OPNb and OPNc, while PTEN splicing isoforms (PTEN-SI) are named vs-fl (full lenght variant), vs-3a, vs-3b, vs-3c, vs-5a, vs-5b, vs-5c, vs-5d and vs-Del E6 (Figure 1 and 2).
- Total OPN is overexpressed in EC and modifications at PTEN gene correspond to one of main the genetic alterations in these tumors. Althought total OPN expression has been correlated to PTEN expression, no data is currently available regarding OPN-SI and their association to PTEN-SI expression.

Table 1: Clinico-pathological characteristics and genetic abnormalities in Type I and II EC_s

Characteristic	Type I (EEC)	Type II (NEEC)
Unopposed oestrogen	Yes	No
Background endometrium	Hyperplastic	Atrophic
Morphology	Endometrioid	Serous, clear cell
Microsatellite instability	20–40%	0–5%
p53 mutations	10–20%	90%
β-Catenin mutations	31–47%	0–3%
K-ras mutations	15–30%	0–5%
PTEN inactivation	35–50%	10%
HER2/neu	No information	18–80%

EEC: endometrial endometrioid carcinoma, NEEC: endometrial non-endometrioid carcinoma. Adapted from Doll *et al.*, (2008)

Figure 1: Osteopontin splicing isoforms. OPNa isoform is the complete splice variant, while OPNb does not contain exon 5 and OPNc lacks exon 4. Adapted from He *et al.*, (2006)

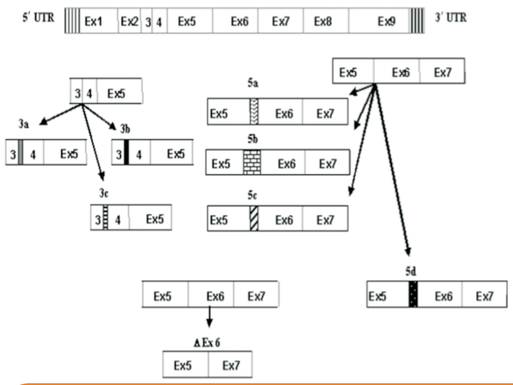


Figure 2: Structure of PTEN splicing isoforms. Full length PTEN (PTEN-FL) isoforms have nine exons. PTEN splicing variants (sv) -3a, -3b, -3c have, respectively, inclusion of 29, 52 and 40 bp after exon 3, while sv-5a, -5b, 5c and 5d variants exhibit, respectively, inclusion of 38, 327, 158 and 60 pb after exon 5. The sv-Del E6 has a deleted exon 6. Adapted from Sarquis *et al.*, (2006).

OBJECTIVE

- Characterize the expression patterns of OPN and PTEN isoforms in EC tumor and non-tumoral cell lines,
- Evaluate investigated isoforms as putative biomarkers for EC,
- Establish putative associations between the expression of OPN and PTEN isoforms in this cell lines.

METHODOLOGY

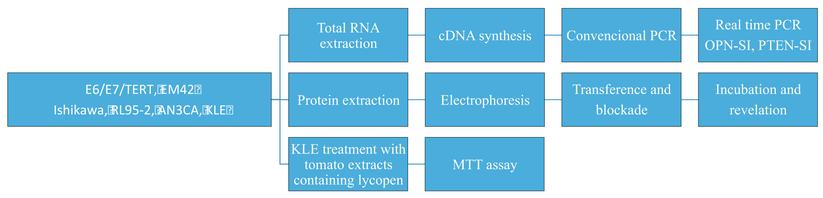


Figure 3. Overall methodology approach to analyze OPN and PTEN isoforms. These isoforms have been analyzed by real time PCR, immunoblot and (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT). All cell lines, except KLE (in which we used DMEM/F12 medium), were cultured in DMEM culture medium. Total RNA was extract using Qiagen Mini-kit and cDNA synthesis was done with Superscript II First-Strand Synthesis system. Then, cDNa samples were analyzed by real time PCR. Immunoblot have been performed using total protein extract from these cell lines. Protein expression analysis has been performed using the O-17 anti-total antibody, anti-PTEN monoclonal antibody, anti-AKT antibody and anti-pAKT antibody. MTT assays were performed using KLE cell line, which was treated for 48, 72 and 96 hs with 0.5-5 mg/ml tomato extracts containing lycopen.

Table 2: Cell line features.

Endometrial cell lines	Subtype/Grade	Mutant PTEN	References
E6/E7/TERT	Non-tumoral	No	Kyo *et al.*, 2003
EM42	Non-tumoral	No	Rong *et al.*, 2002
Ishikawa	Type I / G1	Yes	Myers & Clements, 2001
RL95-2	Type I / G2	Yes	Way *et al.*, 1983
AN3CA	Type I / G3*	Yes	Korets *et al.*, 2014
KLE	Type II / G3*	No	Liu *et al.*, 2014; Korets *et al.*, 2014; ATCC

*Contradictory information, which may be Type I / G3 or Type II / G3

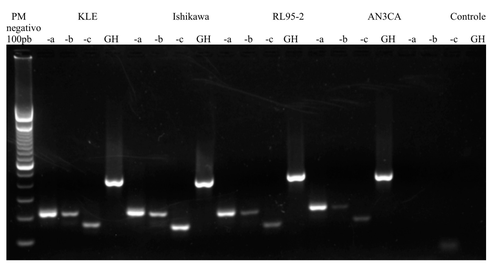


Figure 4: Analysis of the electrophoretic profile of 3 OPN-SI amplicons in KLE, Ishikawa, RL95-2, and AN3CA the cell lines. Representative images of the 3 OPN-SI amplicons corresponding to OPNa, OPNb, and OPNc resulting from RT-PCR amplifications using OPN-SI-specific oligonucleotides. Also shown are the amplicons resulting from the GAPDH constitutive. Amplification products were resolved in **2% agarose gels.** Amplification products exhibit 208pb (OPNa), 209bp (OPNb), 155bp (OPNc) and 418bp (GAPDH). 100bp molecular weight standard. GH GAPDH

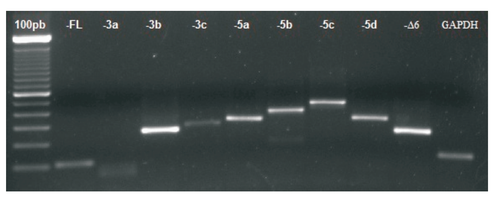


Figure 5: Electrophoretic profile analysis of PTEN isoform amplification products in EM42 cell line. Amplification products of the nine PTEN isoforms (-fl, -3a, -3b, -3c, -5a, -5b, -5c, -5d, -6) are demonstrated using PTEN-SI specific-oligonucleotides. Also shown are the amplicons resulting from the GAPDH constitutive. Amplification products were resolved in **2% agarose gels.** 100bp molecular weight standard.

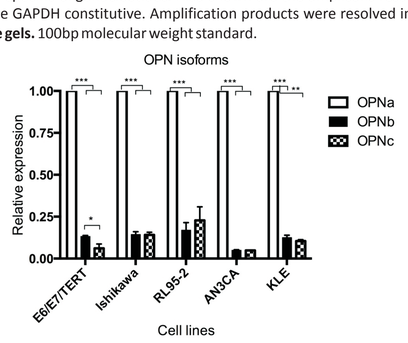
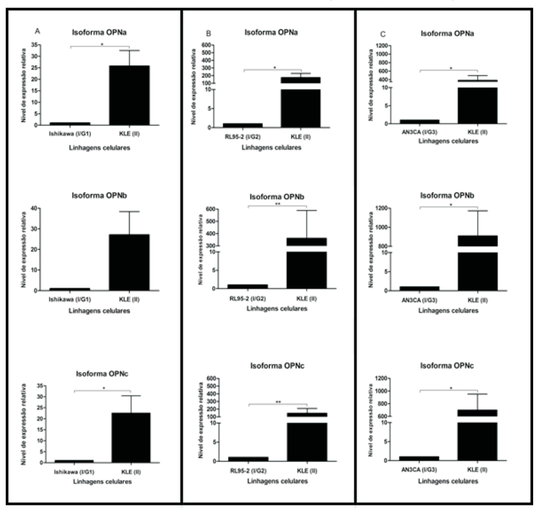


Figure 6. OPN isoforms expression levels in EC and non-tumoral E6/E7/TERT cell lines. The transcriptional levels of the OPN isoforms were analyzed by qRT-PCR, using isoform-specific oligonucleotides. EM42 do not express OPNs. GAPDH was used as constitutive expression control. The results presented were performed in duplicate in 3 independent assays, using the OPNa isoform as reference sample to calculate relative expression levels (reference value = 1). OPNa isoform is expressed in higher levels in relation to OPNb and OPNc isoforms in all cell lines analyzed. *p<0.05, **p<0.01, ***p<0.001

Figure 7. Relative expression level of OPN splicing isoforms in EC cell lines and endometrial non-tumoral cell line E6/E7/TERT. OPN isoforms transcriptional levels were analyzed by qRT-PCR, using isoform-specific oligonucleotides and GAPDH as constitutive expression gene. The results presented were performed in duplicate in 3 independent assays, using the E6/E7/TERT cell line sample as reference sample for relative expression level calculation (reference value = 1). Therefore, the Cts values of OPNa, -b and -c of the E6/E7/TERT were used for calculations of the relative expression levels of each lineage. OPNc isoform has the higher differential expression in all cell lines, between all isoforms. *p<0.05, **p<0.01, 1: expression level of OPNa or OPNb in Ishikawa in relation to RL95-2 (p<0.05) or AN3CA (p< 0.01), 2: expression level of OPNa or OPNb in E6/E7/TERT in relation to RL95-2 (p<0.01) or AN3CA (p<0.0001).



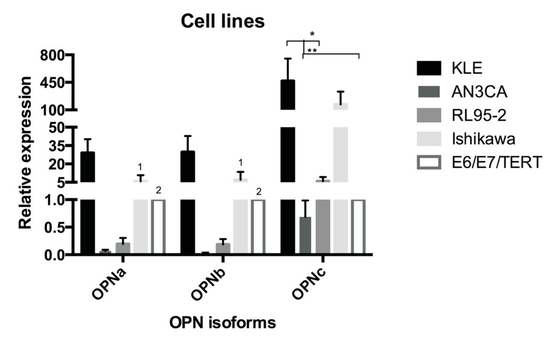


Figure 8. OPN isoforms expression levels in EC cell lines representative of distinct EC tumor grades. The transcriptional levels of the OPN isoforms were analyzed by qRT-PCR, using isoform-specific oligonucleotides. GAPDH was used as constitutive expression control. The results presented were performed in duplicate in 3 independent assays. Transcriptional expression levels of KLE in relation to (A) Ishikawa cell line (I/G1), (B) RL95-2 cell line (I/G2 and (C) AN3CA (I/G3), which were respectively used as reference samples (reference value = 1). KLE cell line exhibit higher expression levels of all three OPN isoforms than other tested cell lines, even when compared to another G3 grade cell line, AN3CA. *p <0.05, ** p <0.01

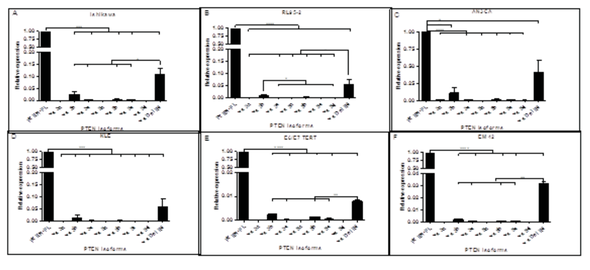


Figure 9: PTEN isoforms expression levels in EC and non-tumoral cell lines. PTEN isoforms transcriptional levels were analyzed by qRT-PCR using isoform-specific oligonucleotides. GAPDH was used as the constitutive gene. The results presented were performed in duplicate, in 3 independent assays. The PTEN isoforms relative expression levels were analyzed in Ishikawa (A), RL95-2 (B), AN3CA (C), KLE (D), E6/E7 TERT (E) and in EM 42 (F) cells, using the full length PTEN as the reference sample. All cell lines tested expressed the nine PTEN splice variants and exhibit similar expression patterns. The full length PTEN isoform is significantly overexpressed in relation to other tested isoforms in all endometrial cell lines tested. Moreover, DEIE6 splice variants is the second predominant isoform. *P <0.05, **P<0.01, ***P<0.001, ****P<0001

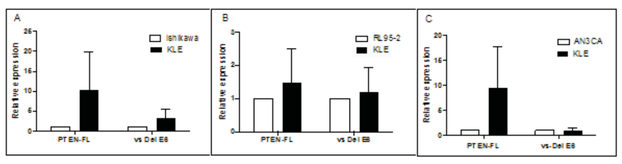
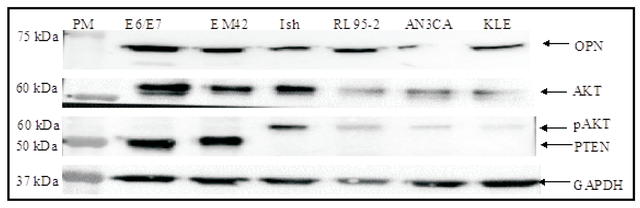


Figure 10: Expression level of full lenght PTEN and Del E6 isoforms in EC cell lines. The transcriptional levels of PTEN-FL and Del E6 isoforms were analyzed by qRT-PCR using isoform-specific oligonucleotides and GAPDH was used as the constitutive gene. The results presented were performed in duplicate in 3 independent assays. (A) Ishikawa, (B) RL95-2 and (C) AN3CA cell lines were used as reference samples. KLE cell line has a higher transcriptional level of PTEN-FL than all the three cell lines representative of Type I EC (Ishikawa, RL95-2 and AN3CA). Moreover, the expression level of vs-Del E6 is also higher in KLE than in Ishikawa and RL95-2 cell line, but similar to AN3CA.

Figure 11: Analysis of protein expression of total OPN, AKT, pAKT, full lenght p53 and PTEN isoforms by immunoblot in EC and endometrial non-tumoral cell lines. The endogenous protein expression of OPN was analyzed using the O-17 anti-total OPN antibody. The expression of AKT and pAKT was analyzed using an specific rabbit antibody diluted 1:1000 and 1:500, respectively. The expression of PTEN-FL was analyzed using a monoclonal PTEN antibody, which recognizes the PTEN carboxi-terminal region. GAPDH expression was used as an endogenous constitutive control. At the protein level, both EC and endometrial non-tumoral cell lines express total OPN and total AKT. However, we can not assert which OPN isoform is being mostly detected using this assay. pAKT is exclusively expressed in tumor cell lines, mostly Ishikawa cells. PTEN-FL is expressed in both analyzed non-tumoral cell lines (E6/E7/TERT and EM42), but not in EC cell lines.



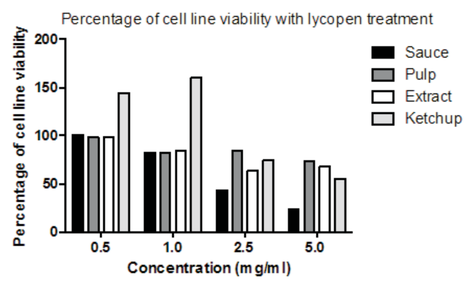


Figure 12: Colorimetric assay with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). 1x105 KLE cells were plated per well, in a 96 well plate. The treatment were done during 48, 72 and 96 hs with 0.5, 1, 2.5 or 5 mg/ml of tomato extracts containing lycopen. The results presented were performed in quadruplicate in 3 independent assays. Treatment of KLE cell line with distinct tomato extracts containing lycopen reduced cell viability, specially using 2.5 mg/ml of sauce extract, which promoted a 50% inhibition on cell viability 96 hs after treatment.

CONCLUSION

- We firstly demonstrated that all OPN and PTEN tested transcript isoforms are expressed in endometrial tumoral and non-tumoral cell lines. Protein expression of the total OPN and PTEN exhibit a distinct expression pattern when compared to transcriptional expression. Total protein OPN is similarly expressed in both tumor and non-tumoral cell lines, while total PTEN is expressed exclusively in non-tumoral cells.
- Our data evidence that full lenght OPN, and PTEN are the major transcripts expressed variants in EC tumor cell lines and in non-tumoral E6/E7/TERT cell line.
- Although our data showed the expression of PTEN-FL transcript is overexpressed in both tested Type I and Type II EC cell lines, as well as in endometrial non-tumoral cells, we did not detected its protein expression in EC cell lines, evidencing a post-transcriptional regulation of these splice variants.
- Our data provide early evidence that these OPN and PTEN isoforms could differently modulate the expression and functional roles of their full-length counterparts. Once some of these isoforms display differential expression between endometrial tumoral and non-tumoral cell lines, as well as between cell lines representative of distinct EC tumor grades, we then provide some indications that some of these transcript isoforms could be considered as potential EC biomarkers. Further work should test their potential application as biomarkers and how OPN isoforms levels could modulate PTEN isoforms expression patterns and tumor suppressive roles.

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