**KLK10 exon 3 unmethylated PCR product concentration: a new potential early diagnostic marker in ovarian cancer? - A pilot study**

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**Abstract**

**Background**: KLK10 exon 3 hypermethylation correlated to tumor-specific lack of KLK10 expression in cancer cell lines and primary tumors. In the present study we investigate the possible role of KLK10 exon 3 methylation in ovarian tumor diagnosis and prognosis.

**Results**: Qualitative methylation-specific PCR (MSP) results did not show statistically significant differences in patient group samples (normal and tumor) where all samples were positive only for the unmethylated-specific PCR except for two malignant samples that were either doubly positive (serous carcinoma) or doubly negative (Sertoli-Leydig cell tumor) for the two MSP tests. However, KLK10 exon 3 unmethylated PCR product concentration (ng/μl) showed statistically significant differences in benign and malignant patient group samples; mean ± SD (n): tumor: 0.077 ± 0.035 (14) and 0.047 ± 0.021 (15), respectively, \( p \)-value = 0.011; and normal: 0.094 ± 0.039 (7) and 0.046 ± 0.027 (6), respectively, \( p \)-value = 0.031. Moreover, ROC curve analysis of KLK10 exon 3 unmethylated PCR product concentration in overall patient group samples showed good diagnostic ability (AUC = 0.778; \( p \)-value = 0.002). Patient survival (living and died) showed statistically significant difference according to preoperative serum CA125 concentration (U/ml); median (n): 101.25 (10) and 1252 (5), respectively, \( p \)-value = 0.037, but not KLK10 exon 3 unmethylated PCR product concentration (ng/μl) in overall malignant patient samples; mean ± SD (n): 0.042 ± 0.015 (14) and 0.055 ± 0.032 (7), \( p \)-value = 0.228.

**Conclusion**: To the best of our knowledge, this is the first report on KLK10 exon 3 unmethylated PCR product concentration as potential early epigenetic diagnostic marker in primary ovarian tumors. Taken into account the limitations in our study (small sample size and semi-quantitative PCR product analysis) further studies are strongly recommended.

**Keywords**: Ovarian cancer, CA125, KLK10, KLK6, KLK10 exon 3 methylation

**Background**

DNA methylation is one of the well-studied epigenetic modifications in DNA/chromatin metabolism. It is a dynamic process and involves the reversible and heritable methylation of the 5′ carbon of cytosine residues to yield 5-methylcytosine (5-MC) [1]. The reaction belongs to one carbon metabolism where DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are the biocatalysts and S-adenosylmethionine (SAM) is the methyl-donor [2]. Besides having important physiological roles in cell differentiation, development and gene regulation [3], DNA methylation can provide clues to other physiological processes, e.g. cell and tissue aging [4] and establishment of memory [5].

In DNA the cytosine residues occur either in frequencies that are far less than expected or in CpG-rich short stretches (0.5–4 kbp) in gene promoters and other regulatory regions known collectively as CpG islands...
with sets KLK10 and in primary tumors [23] nant samples; but not normal ones, in cell lines [23] of ovarian tumors. Possible role in the biology, diagnosis and/or prognosis KLK10 may be understood as KLK10 confinement of KLK10 study, [24]. Results of previous works were consistent about the shown to perfectly follow that of breast cancer [23]. The rationale behind such correlation methylation in a majority of cell lines and in primary cancers [12]. Indeed, tumor-specific lack of KLK10 expression correlated to KLK10 exon 3 hypermethylation in a majority of cell lines and in primary breast cancer [23]. The rationale behind such correlation may be understood as KLK10 exon 3 methylation was shown to perfectly follow that of KLK10 gene promoter [24]. Results of previous works were consistent about the confinement of KLK10 exon 3 hypermethylation in malignant samples; but not normal ones, in cell lines [23–25] and in primary tumors [23–26]. However, in some sample sets KLK10 expression did not show simple correlation with KLK10 exon 3 methylation [23–26]. In the present study, KLK10 exon 3 methylation is assessed for its possible role in the biology, diagnosis and/or prognosis of ovarian tumors.

Methods

Patients and samples

This is a further study for our previous work on serum KLK6 and 10 in ovarian cancer patients [27]. The protocol of the present study was approved by the Ethics and Research Committee of Ain Shams University.

Patients’ demographic data and preoperative serum marker levels (CA125, KLK10 and KLK6) were taken from our previous data as mentioned above. Patients or their relatives were contacted from October 2012 to January 2013 to collect patient follow-up data and have their written informed consent. The studied samples were archival FFPE-ovarian tissue samples that are available at the Gynecologic Oncology Unit, Ain Shams University Maternity Hospital. The included samples were tumors and their contralateral normal ovarian samples (for some cases), while non-neoplastic ovarian masses, i.e. inflammation and endometriosis, were excluded.

Experimental protocol

Using histopathology microtome (microTec® cut 4050, UK) 10 to 15 tissue sections, each of ~ 2 × 2 cm in diameter and 1–2 μm thickness were cut and put in clean and autoclaved 1.5 ml microcentrifuge tube, one day before DNA extraction. An H&E stained tissue section was prepared and examined microscopically for each sample.

DNA extraction

Commercially available QIAamp DNA FFPE Tissue Kit (Qiagen) was used. The steps of the experiment were done according to the product insert provided by the manufacturer. Xylol (Sigma-Aldrich, Germany) was used to dissolve the paraffin and 98–100% ethanol (Sigma-Aldrich, Germany) was used to remove the xylol and to reconstitute the buffers. The concentration of the extracted pure DNA was measured spectrophotometrically (Ultraspec® 1000, Amersham Pharmacia Biotech, Cambridge, England) at 260 nm and the DNA samples were stored at −80 °C until the time of DNA sodium bisulfite treatment.

Sodium bisulfite treatment of DNA

One μg of extracted DNA was treated by sodium bisulfite using commercially available EpiTect Bisulfite Kit (Qiagen). The steps of the experiment and the thermal cycler program were done according to the product insert given by the manufacturer for FFPE-samples. The sodium bisulfite converted DNA samples were kept at −80 °C until the time of MSP experiment.

MSP for KLK10 exon 3

Commercially available HotStarTaq Master Mix kit (Qiagen) was used. Methylation- and unmethylated-specific PCRs were run in parallel in separate PCR tubes where each tube contained either methylated- or unmethylated-specific primer pair, respectively. The two primer pairs were provided by Invitrogen (USA), as described elsewhere [23]. Each PCR reaction contained ~ 0.5 μg sodium bisulfite-treated DNA sample. The concentrations of the PCR reaction components were made according to the manufacturer’s instructions in the product insert. A methylation positive control MSP was done using 2 μg (1 μg for each MSP test type) fully methylated DNA (EpiTect® Control DNA (human), methylated and bisulfite converted (100), Qiagen). The thermal cycler program was as follows: 1 cycle of 95 °C for 15 min; 35 cycles of
94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and 1 cycle of 72 °C for 10 min.

Capillary gel electrophoresis
PCR products were assessed by capillary gel electrophoresis using QIAxcel system (Qiagen). A QIAxcel kit (QIAxcel DNA High Resolution Kit (1200)) was used and the experiment steps were done according to the manufacturer’s instructions in the product insert. Determination of DNA fragment size and concentration was done using the BioCalculator software provided with the QIAxcel (Qiagen).

Statistical analysis
Statistical analysis of data was done using SPSS (version 15.0 for Windows). p < 0.05 was considered the cutoff value for significance. Chi-square test ($\chi^2$) was used to test the association of categorical data. Parametric or non-parametric tests were used to compare mean ± SD (Student t-test or ANOVA test) or median and interquartile range (Mann-Whitney test or Kruskal Wallis test) in two or more populations, respectively, involving independent samples. Spearman’s test was used to evaluate correlations between continuous variables. A receiver operating characteristic (ROC) curve was used to illustrate the diagnostic properties of a test on a numerical scale. Marker’s diagnostic parameters were calculated as follows: sensitivity = true positive tests/ all positive samples by gold standard test; positive predictive value (PPV) = true positive tests/ all positive tests; specificity = true negative tests/ all negative samples by gold standard test; negative predictive value (NPV) = true negative tests/ all negative samples; and accuracy = true positive and true negative tests/all positive and all negative tests.

Results
Patients and samples
This study included 14 benign and 16 malignant ovarian tumor patients. Patient clinicopathological data are shown in Table 1. There were no statistically significant differences of patient age mean ± SD according to disease grade (1, 2 and 3): mean ± SD (n): 36.8 ± 11.2 (5), 48.0 ± 19.6 (5) and 46.5 ± 11.0 (6), respectively; p-value = 0.422, or disease stage (I/II and III/IV): mean ± SD (n): 42.5 ± 16.4 (9) and 45.7 ± 11.7 (7), respectively; p-value = 0.675.

Each patient participated by one tumor sample. In addition, samples from contralateral normal ovaries of some patients in benign and malignant patient groups were included as normal controls (n = 7 and 6, respectively). Table 2 shows the pathological characteristics of tumor samples in the two patient groups. Histopathological H&E examination of all samples showed good quality of the tissue samples and confirmed the previously known diagnoses.

Table 1 Clinicopathological parameters of patient groups

| Patient group | Benign (N = 14) | Malignant (N = 16) | p-value |
|---------------|-----------------|-------------------|---------|
| Age (mean ± SD) | 45.2 ± 12.7 | 43.9 ± 14.2 | 0.788c |
| Menstrual cycle | | | |
| Premenopausal | 7 | 11 | 0.296a |
| Postmenopausal | 7 | 5 | 0.011a |
| Ascites | | | |
| Absent | 13 | 8 | 0.011a |
| Present | 1 | 8 | 0.011a |
| Serum marker (median) | | | |
| CA125 (U/ml) | 11.6 | 186.0 | 0.000b |
| KLK10 (ng/ml) | 1.41 | 1.99 | 0.236b |
| KLK6 (ng/ml) | 3.05 | 3.17 | 0.678b |

MSP
In the overall there were 42 unmethylated-specific positive PCR tests and only one methylated-specific positive PCR test. The unmethylated PCR band size was as follows: min.-max.: 101.30–274.00 bp; median: 123.45 bp and mean ± SD: 126.25 ± 23.61 bp, whereas the methylated band was 134.5 bp. The methylation positive control sample was positive for the methylated-specific PCR (band size: 129.0 bp) but negative for unmethylated-specific PCR.

Qualitative results of MSP did not show statistically significant difference in the two patient groups for both tumor and normal samples (Table 3). However, comparison of KLK10 exon 3 unmethylated PCR product concentration mean ± SD (ng/μl) showed statistically significant differences in benign (n = 14), malignant (n = 15) and overall normal (n = 13) samples; mean ± SD: 0.077 ± 0.035, 0.047 ± 0.021, and 0.072 ± 0.041, respectively; p-value = 0.046. Moreover, KLK10 exon 3 unmethylated PCR product concentration mean ± SD (ng/μl) showed statistically significant differences in tumor and normal samples in the two patient groups (Fig. 1).

Spearman’s correlation of KLK10 exon 3 unmethylated PCR product concentration (ng/μl) showed statistically significant positive correlation with patient age (p = 0.398; p-value = 0.009) and statistically significant negative correlation with serum KLK10 (p = −0.306; p-value = 0.054) but not KLK6 (p = −0.114; p-value = 0.471) or CA125 (p = −0.214; p-value = 0.197). Comparisons of KLK10 exon 3 unmethylated PCR product concentration mean ± SD (ng/μl) in overall samples and in sample pathology types in the two patient groups according to patient age and serum KLK10 are shown in Tables 4 and 5, respectively.
ROC curves

ROC curves for KLK10 exon 3 unmethylated PCR product concentration (ng/µl) and preoperative serum markers in the two patient groups are shown in Figs. 2a and b, respectively.

Combination of KLK10 exon 3 unmethylated PCR product concentration and serum marker showed enhanced combined marker sensitivity with all 3 markers and better specificity with CA125 but not KLK10 or KLK6. Combined

Table 2 Pathological characteristics of tumor samples

| Pathology subgroup/subtype | Benign tumors (N, %) | Malignant tumors (N, %) |
|----------------------------|----------------------|-------------------------|
| Neoplastic (14, 100%)       | Epithelial (11, 68.75%) |
| Serous cystadenoma (3)      | Serous (4)            |
| Serous cystadenofibroma (1) | Mucinous (2)          |
| Simple serous cyst (2)      | Endometrioid (4)      |
| Granulosa cell tumor (3)    | Clear cell (1)        |
| Mature cystic teratoma (3)  | Non-epithelial (3, 18.75%) |
| Mucinous cystadenoma (1)    | Yolk sac tumor (1)    |
| Brenner tumor (1)           | Liposarcoma (1)       |
| Sertoli-leydig cell tumor (1)| Colon cancer (2)     |

| Grade | Benign | Malignant |
|-------|--------|-----------|
| 1     | 5 (31.3%)    | 5 (31.3%) |
| 2     | –       | 6 (37.5%) |
| 3     | –       | –         |

| Stage | Benign | Malignant |
|-------|--------|-----------|
| I/II  | 9 (56.3%) | –         |
| III/IV| 7 (43.8%) | –         |

Table 3 MSP qualitative results in patient group samples

| MSP | Normal samples | Tumor samples |
|-----|----------------|---------------|
|     | Benign patient | Malignant patient | Benign patient | Malignant patient |
| Methylated-specific PCR | | | | |
| Positive | 0 | 0 | 0 | 1b |
| Negative | 7 | 6 | 14 | 15 |
| p-value | – | 0.341a | | |
| Unmethylated-specific PCR | | | | |
| Positive | 7 | 6 | 14 | 15 |
| Negative | 0 | 0 | 0 | 1b |
| p-value | – | 0.341a | | |

- not applicable

*a*χ² test

*b*The two odd results in the malignant samples belonged to two samples; one was doubly positive (ovarian serous adenocarcinoma) and the other was doubly negative (Sertoli-Leydig cell tumor) for the two MSP test types

marker profile of KLK10 exon 3 unmethylated PCR product concentration with serum marker was as follows; CA125: sensitivity: 100%, specificity: 94.4%, PPV: 68.9%, NPV: 73.9% and accuracy: 71.1%; KLK10: sensitivity: 90.4%, specificity: 85.7%, PPV: 67.8%, NPV: 62% and accuracy: 64.9%; and KLK6: sensitivity: 85.7%, specificity: 71.4%, PPV: 60%, NPV: 60% and accuracy: 60%.
Table 4: Comparison of KLK10 exon 3 unmethylated PCR product concentration (ng/μl) according to patient age

| KLK10 exon 3 unmethylated PCR product; ng/μl, mean ± SD | Age group | p-value |
|--------------------------------------------------------|-----------|---------|
| Overall samples (n = 6–27)                              | 16–32 years | 33–55 years | > 55 years |
| Benign patients:                                         |           |           |           |
| Normal ovary (n = 0–5)                                   |           |           |           |
| Benign ovary (n = 2–10)                                  |           |           |           |
| p-value                                                  |           |           |           |
| Malignant patients:                                      |           |           |           |
| Normal ovary (n = 1–3)                                   |           |           |           |
| Malignant ovary (n = 3–9)                                |           |           |           |
| p-value                                                  |           |           |           |

- no samples or statistical test not applicable, SD standard deviation

*Student t-test

Table 5: Comparison of KLK10 exon 3 unmethylated PCR product concentration (ng/μl) according to preoperative serum KLK10

| KLK10 exon 3 unmethylated PCR product; ng/μl, mean ± SD | Serum KLK10 | p-value |
|--------------------------------------------------------|-------------|---------|
| Overall samples (n = 15–27)                             | < 2 ng/ml    | ≥ 2 ng/ml | 0.007** |
| Benign patients:                                         |             |           |         |
| Normal ovary (n = 1–6)                                   | 0.100 ± 0.040 | 0.060 ± – | 0.397** |
| Benign ovary (n = 3–11)                                  | 0.086 ± 0.033 | 0.043 ± 0.023 | 0.060** |
| p-value                                                  | 0.463*      | 0.596*   |         |
| Malignant patients:                                      |             |           |         |
| Normal ovary (n = 2–4)                                   | 0.070 ± 0.042 | 0.035 ± 0.010 | 0.153* |
| Malignant ovary (n = 7–8)                                | 0.044 ± 0.023 | 0.051 ± 0.019 | 0.511* |
| p-value                                                  | 0.252*      | 0.156*   |         |

- not applicable, SD standard deviation

*Student t-test

Patients’ survival

Five-year patient follow-up data were available between 2007 and 2012. Five patients died within the first two years of the follow-up period. In the living 11 patients there were 9 disease-free and 2 relapsed. Comparisons of patient clinicopathological parameters according to patient survival (living and died) showed statistically significant differences in preoperative serum CA125 concentration (U/ml); median (n): 101.25 (10) and 1252 (5), respectively; p-value = 0.037, and disease stage (I/II and III/IV): late stage was more frequent in died patients compared to the living (4 vs. 1, respectively); p-value = 0.049. The p-values for other clinicopathological parameters were as follows: patient age mean ± SD: 0.355; serum KLK10 (ng/ml) mean ± SD: 0.798; serum KLK6 (ng/ml) mean ± SD: 0.278; ascites (absent and present): 0.106; disease grade (1, 2, and 3): 0.622; pathology subgroup (epithelial, nonepithelial and metastatic): 0.402; and epithelial pathology subtype (serous, mucinous, endometrioid and clear cell carcinoma): 0.735.

Comparison of KLK10 exon 3 unmethylated PCR product concentration (ng/μl) mean ± SD according to patient survival (living and died) did not show statistically significant differences in overall malignant patient samples: mean ± SD (n): 0.042 ± 0.015 (14) and 0.055 ± 0.032 (7), respectively; p-value = 0.228, normal samples: mean ± SD (n): 0.035 ± 0.010 (4) and 0.070 ± 0.042 (2), respectively; p-value = 0.153, or malignant samples: mean ± SD (n): 0.046 ± 0.017 (10) and 0.050 ± 0.030 (5), respectively; p = 0.748. However, the oddly methylated-specific PCR positive sample in our results was serous carcinoma of grade 3, stage III/IV and its unmethylated PCR product concentration was 0.06 ng/μl; the patient of which died one year after diagnosis and start of treatment.

Discussion

In the literature we could find only one study on KLK10 exon 3 methylation in primary ovarian tumors that was reported by Sidiropoulos and colleagues [25]. Our results agree with those in the previous work with some logic approximation due to differences in the used technique for gene methylation assessment (MSP vs. direct DNA sequencing, respectively) and the examined KLK10 expression parameter (serum KLK10 vs. cytosolic KLK10, respectively). The two studies showed KLK10 exon 3 methylation only in malignant samples (methylated-specific PCR positive samples: 1/16 and samples with partially methylated KLK10 exon 3: 6/7, respectively) but not in normal samples (methylated-specific PCR negative samples: 13/13 and samples with fully unmethylated KLK10 exon 3: 2/2, respectively). Additionally, we studied KLK10 exon 3 methylation in benign ovarian tumors – not included in the previous work – and they all were unmethylated (Table 3). Differences in the internal working of MSP and direct DNA
sequencing would make comparison of frequencies of methylated samples in the two studies practically unfeasible (see later).

Moreover, as for the correlation between KLK10 gene expression and KLK10 exon 3 methylation the two studies showed similar results. In the previous work there was fair KLK10 expression in normal samples (fully unmethylated) and high expression in the partially methylated malignant samples (3 out of 6). In concordance to those results our samples showed negative correlation between serum KLK10 level (ng/ml) and KLK10 exon 3 unmethylated PCR product concentration (ng/μl) by Spearman’s correlation and by comparing the unmethylated PCR product concentration mean ± SD in overall samples according to KLK10 level (Table 5).

While our qualitative MSP results did not show statistically significant differences in patient group samples (Table 3), KLK10 exon 3 unmethylated PCR product concentration (ng/μl) mean ± SD showed statistically significant differences in normal and tumor samples in the two patient groups (Fig. 1). Because our MSP experiment conditions were almost constant for all PCR tests (including the operator, instruments and machines, kits and reagents, DNA template concentration, PCR program, simultaneous unmethylated- and methylated-specific PCRs, and random sample sets in 4 PCR runs done in 4 different days) those statistically significant differences of KLK10 exon 3 unmethylated PCR product concentration should be due to differences in the methylation patterns of samples rather than due to unfounded chance (see later).

Individual cells in the same tissue may show heterogeneity as regard their DNA methylation patterns [28]. Indeed, understanding that methylation pattern heterogeneity is fundamental in interpreting qualitative and quantitative DNA methylation data and also for appreciating and understanding several aspects of gene functions. Each cell contains two copies of each CpG site; one on each of the two homologous chromosomes, and, therefore, can have on its own 3 different methylation patterns for that particular CpG site: fully unmethylated, fully

|                      | Best cutoff | Area under the curve | p-value | Sensitivity | Specificity | PPV | NPV | Accuracy |
|----------------------|-------------|-----------------------|---------|-------------|-------------|-----|-----|----------|
| KLK10 exon 3 unmethylated PCR product (ng/μl) | 0.065       | 0.778                 | 0.002   | 61.9%       | 85.7%      | 69.2% | 81.2% | 73.8%    |
| CA125 (U/ml)         | 26.85       | 0.894                 | 0.001   | 80%         | 83.3%      | 85.7% | 76.9% | 81.4%    |
| KLK10 (ng/ml)        | 2           | 0.627                 | 0.236   | 50%         | 78.6%      | 72.7% | 57.8% | 62.06%   |
| KLK6 (ng/ml)         | 3.11        | 0.545                 | 0.678   | 56.3%       | 57.1%      | 60%  | 53.3% | 56.6%    |
methylated or partially methylated. The possible methylation patterns of a number \(n\) of CpG sites are given mathematically as \(2^n\). For example, when studying an average number of 12 CpGs by MSP, the possible methylation patterns in the DNA sample are \(2^{12} = 4096\), which reflects possible immense methylation pattern heterogeneity when the cell can have any two of these patterns. However, in the context of \(KLK10\) exon 3 in ovarian cancer the property of methylation pattern heterogeneity would be present almost exclusively in malignant samples but not in normal ones because in the previous work the malignant samples were partially methylated, whereas the normal samples were fully unmethylated [25].

In Fig. 3 we present a theoretical rationale for qualitative and quantitative MSP results based on possible methylation pattern heterogeneity in individual cells of a sample [28], the independency of the primer pairs in MSP from one another [23, 29] and previous DNA methylation results by MSP and direct DNA sequencing in cell lines and primary breast cancer [23]. We hypothesize that the existence of different partially methylated patterns in the sample would result in the stratification of the starting DNA template into template classes with varying MSP-

**Fig. 3** The figure shows a schematic representation of a theoretical rationale for MSP results based on previous works [23, 28, 29]. The DNA templates (long bars with 3′ → 5′ directionality designation) show blue and red dots representing unmethylated and methylated CpGs, respectively. The short blue and red bars represent unmethylated- and methylated-specific primer pairs, respectively. For simplicity, we show methylation patterns with uniform methylation status at the primer’s annealing site on DNA template (a–h) and only two with non-uniform methylation status (i and j). While fully unmethylated (a) and fully methylated (b) patterns provide unmethylated- and methylated-specific positive PCRs, respectively, partially methylated patterns (c–j) can have any of the four possible qualitative MSP results. MSP-inexpressible partially methylated pattern (d) may rationalize our doubly negative malignant sample (Sertoli-Leydig cell tumor), in addition to possible mutation as methylated CpGs are known hotspot for mutations [39, 40].
expressivities according to the number of methylation-wise matching CpG sites with their corresponding nucleotides in the respective PCR primer. While some patterns would be more or less favorable for primer annealing (Fig. 3: elements C, and E–J), other patterns may not be at all (Fig. 3: element D). Differences of the proportions of those differentially MSP-expressible partially methylated patterns in the samples may be effected practically as differences in the unmethylated PCR product concentration. Given that those partially methylated patterns would occur only in malignant samples but not in normal – and probably also benign – samples (see above), such proportionate decreases in the unmethylated PCR product concentration would happen in malignant samples only.

Our results showed statistically significant positive correlation between KLK10 exon 3 unmethylated PCR product concentration (ng/μl) and patient age by Spearman’s correlation and on comparing its mean ± SD in overall samples and in malignant samples according to age groups (Table 4). However, because there was no statistically significant difference of patient age in the two patient groups (Table 1), comparison of KLK10 exon 3 unmethylated PCR product concentration in patient group samples should be valid and justifiable.

KLK10 exon 3 methylation correlated to high disease grade (breast cancer [30]), late stage (lung cancer [26]) and bad patient prognosis (acute lymphoblastic leukemia [24]). Similarly, the only methylated-specific PCR positive sample in our results was serous carcinoma of high grade (3) and late stage (III/IV) whose patient died within one year after starting the treatment. However, in our results comparison of KLK10 exon 3 unmethylated PCR product concentration (ng/μl) according to disease grade, stage or patient survival did not show statistically significant differences that may be attributable to early implication of deregulated KLK10 exon 3 methylation in the disease or lack of significant information about site-specific CpG methylation in MSP data (see later).

The notion that information about the exact location of DNA methylation maybe crucial for correct clues about disease diagnosis, prognosis and gene function [31] would greatly restrain the translational impact of DNA methylation data by MSP. For example, lack of site-specific CpG methylation data (in MSP) and/or data about their pattern distribution in the sample (in MSP and direct DNA sequencing, respectively) may explain – at least in part – the inconsistent results, in sample subsets or sample types, about the downregulation of KLK10 through KLK10 exon 3 hypermethylation in previous works [23–26]. At the functional aspect, the reported antitumor role of KLK10 was demonstrated in vector-mediated transfection experiments in vitro and/or in tumor xenograft in mice using different cancer cell lines; e.g. breast [32], prostate [16] and gastric cell [33], including ovarian cell line [34].

Noteworthy is that overexpression of KLK10 was found in malignant sample subsets (tissues or sera) in cancer types in which KLK10 was shown experimentally as tumor suppressor; breast [35], prostate [19], gastric [36] and ovary [20,27]. Taken together, the antitumor role of KLK10 may be understood only in relation to the context of the given study as regard type of tissue and the underlying pathphysiologic processes [37].

Indeed, the translational impact of DNA methylation data can vary greatly with the pros and cons of the used tools. While MSP studies only a few of CpGs without regard to their exact location, direct DNA sequencing can give site-specific CpG methylation data for all CpGs of interest as percent methylation in the sample. Unfortunately, both tools fall short of providing information about site-specific CpG methylation pattern distribution in sample. The more recently discovered next generation sequencing have the advantage of presenting site-specific DNA methylation data in absolute numbers and may tackle the problem of methylation pattern distribution in hand of sophisticated computational methods [38]. Nonetheless, considering possible dynamicity of DNA methylation as a mechanism for gene regulation [1] another dimension for DNA methylation pattern resolution may be necessary in order to spot and trace such spatiotemporal DNA methylation events rather than making statements based merely on single static snapshot image.

Conclusions
In summary, our results agreed with those in the previous work as for the exclusive occurrence of KLK10 exon 3 methylation in malignant but not in normal ovarian samples. Additionally, we assessed KLK10 exon 3 methylation in benign ovarian tumors (not included in the previous work) and found that they were unmethylated. Although our qualitative MSP results did not show statistically significant differences in patient group samples, semi-quantitative KLK10 exon 3 unmethylated PCR product concentration (ng/μl) showed statistically significant differences not only in tumor samples but also in otherwise histologically normal samples in benign and malignant patients. These findings would suggest, for the first time, KLK10 exon 3 unmethylated PCR product concentration as potential early epigenetic diagnostic marker in ovarian cancer. The diagnostic potential of KLK10 exon 3 unmethylated PCR product concentration in overall patient group samples was also evident in ROC curve analysis, and it could enhance the sensitivity of preoperative serum markers CA125, KLK10 and KLK6. Taking into account the limitations in the present study (small sample size and semi-quantitative assessment of PCR product concentration) further studies are needed before making a statement about the validity and reproducibility of these results. Moreover, we highlighted the translational
limitations of DNA methylation data in MSP and direct DNA sequencing and addressed future challenges of the more recent next generation sequencing (NGS) to further enhance the translational impact of DNA methylation data.

Abbreviations
S-MC: 5-methylcytosine; bp: Base pair; CA125: Cancer antigen 125; DNA: Deoxyribonucleic acid; DNMT1: DNA methyltransferase 1; DNMT3a: DNA methyltransferase 3a; DNMT3b: DNA methyltransferase 3b; FFPE: Formalin-fixed paraffin-embedded; H&E: Hematoxylin and eosin; KLK10: Kallikrein-related peptidase 10; KLK10: Kallikrein-related peptidase 10 gene; KLK6: Kallikrein-related peptidase 6; MECs: Human mammary epithelial cells; microRNA: micro RNA; MSP: Methylation-specific PCR; NEC: Human normal epithelial cell line; NGS: Next generation sequencing; NPV: Negative predictive value; PCR: Polymerase chain reaction; PPV: Positive predictive value; SAM: S-adenosylmethionine

Acknowledgments
We thank Dr. Mohamed Badr (PhD) from the Medical Research Center, Ain Shams Faculty of Medicine for helping in DNA extraction. We thank also Drs. Nasimina Khazagyi (PhD, MD), Nareas Mahmoud (PhD), Fatma Shehata (PhD) and Asila Hussein (PhD) from the Oncology Diagnostic Unit, Biochemistry Department, Ain Shams Faculty of Medicine for helping in MSP experiments. Our thanks are due to Colors for Research laboratory in Cairo for conducting the capillary gel electrophoresis experiments. We are also much indebted to Drs. Hala El Sallaly (PhD, MD) and Sahar Ezz Elarab (PhD, MD) for helping in histopathology grading and in the statistical analysis of data, respectively.

Availability of data and materials
All data generated or analyzed during this study were included in this published article.

Authors’ contributions
MAE and AAM contributed equally to the conception and design of this work. MAE collected patient data and tissue samples and performed the experiments. ZAS performed and examined H&E sections of tissue samples. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The protocol of the present study was approved by the Ethics and Research Committee of Ain Shams University. An informed written consent was taken from the living participants and relatives of died patients.

Competing interests
The authors declare that they have no competing interests.

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Received: 18 January 2018 Accepted: 17 April 2018
Published online: 24 April 2018
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