International Journal of
Cancer Research

Human Epididymis Protein 4 (HE4) mRNA as a Prognostic Marker in Ovarian Tumors in Relation to RMI and CA125

[1]Manar Yehia, [1]Amal Mansour and [2]Sherif Mekawy

[1]Department of Medical Biochemistry and Molecular Biology, Ain Shams University, Abbassia, Cairo, 11381, Egypt

[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Ain Shams University, Abbassia, Cairo, 11381, Egypt

Corresponding Author: Amal Mansour, Department of Medical Biochemistry and Molecular Biology, Ain Shams University, Abbassia, Cairo, 11381, Egypt Tel: +20201111192610

ABSTRACT

Human Epididymis protein 4 (HE4) has recently been shown to improve the sensitivity and specificity of Epithelial Ovarian Cancer (EOC) diagnosis but its function in cancer cells is not clear. We evaluated HE4 expression, RMI and CA125 serum level as diagnostic tools of primary ovarian cancer in Egyptian women. The HE4 gene expression was evaluated by real time PCR in ovarian cancer of 50 Egyptian women. Ovarian cancer tissues were studied for the detection of the gene expression of HE4 by Quantitative Real Time PCR (Q RT-PCR). Serum Human cancer antigen 125 (CA 125) was measured in the serum of all participants of the study using immune sorbent assay (ELISA). The HE4 showed significant difference among ovarian malignant tumors patients compared to the control subjects (p<0.01). The best cutoff value 0.053 at which HE4 sensitivity was 92% and specificity was 96%. There was a significance correlation between HE4, RMI and CA125 in all patients of the study (p≤0.01 for both). The mRNA expression of HE4 was significantly high versus the control group in early stages and low grades of the disease (p = 0.00, 0.01, respectively). As well as, there was Increased HE4 expression in the late stages of the disease suggesting that it may be associated with poor prognosis as well. The HE4 could be considered as a good prognostic marker for ovarian cancer that increases the sensitivity of the CA 125 to absolute value without affection of CA125 accuracy and its positive predictive value.

Key words: Cancer, RMI, ovary, real time PCR, CA 125

INTRODUCTION

Ovarian cancer is a lethal gynecologic malignancy with greater than 70% of women presenting with advanced stage disease (Lu *et al.*, 2013). Worldwide it is estimated that there are 225500 new cases of ovarian cancer and 140200 deaths every year including 14030 deaths in the United States alone (Ferlay *et al.*, 2010; Siegel *et al.*, 2013).

Despite new treatments, long term outcomes have not significantly changed in the past 30 years with the five year overall survival remaining between 30 and 40% (Siegel *et al.*, 2013). Greater than 60% of advanced stage patients will develop recurrent disease (Salani *et al.*, 2007). Patients with advanced stage disease have a five year overall survival between 20 and 40%, in stark contrast to the greater than 90% five year overall survival of patients identified and treated with stage I disease (Cannistra, 2004).

Given the poor prognosis for patients with advanced stage disease, effective screening modalities are needed to identify patients with early stage disease. The majority of women with

early stage disease are asymptomatic and unfortunately when they do present for diagnosis, three quarters are found to have regional or distant metastases (Holschneider and Berek, 2000).

Tumorigenesis is an early event associated with malignant transformation and tumor growth promotion, a process more closely associated with tumor growth and metastasis, Some tumorigenic factors contribute to malignant transformation but not necessarily tumor growth and/or metastasis and vice versa (Li *et al.*, 2013). Genetic and hormonal alterations are considered two major etiological factors for ovarian cancer development. Many oncogenes achieve a high level of expression via genetic amplification. In humans, HE4, SLPI and several other WAP members co-locate in 20q (Bouchard *et al.*, 2006) a region frequently amplified in a variety of cancers (Van Dekken *et al.*, 2001). HE4, a putative protease inhibitor containing two WAP (Whey Acid Protein) domains (Li *et al.*, 2013) has been used widely for the early screening (Winstead, 2009; Scholler *et al.*, 2006) and for differential diagnosis (Huhtinen *et al.*, 2009; Montagnana *et al.*, 2009) of ovarian cancer, as well as for monitoring disease recurrence (Anastasi *et al.*, 2010) and progression (Kobel *et al.*, 2008). Its role in tumorogenesis is investigated by forced over expression of HE4 that promoted several malignant phenotypes including cell proliferation, cell invasion capability, anchorage independent growth and increased tumor growth (Li *et al.*, 2013).

The RMI was developed with the goal of identifying patients at high risk of harboring an ovarian malignancy in order to triage patients accurately to specialty care. First described by Jacobs *et al.* (1990), RMI is the product of CA125 (U mL^{-1}), the ultrasound result (expressed as 0, 1, 3 or 4) and menopausal status (1 if premenopausal and 3 if postmenopausal) (Jacobs *et al.*, 1990). The aim of this study is to ascertain HE4 clinical utility to do a comprehensive assessment of HE4 protein expression in benign and malignant ovarian tissues by real time PCR and its relation to CA125 and RMI that may have predictive and therapeutic impact on patients.

MATERIALS AND METHODS

Participants: This case-control study was performed on 50 female patients with ovarian mass, who were admitted to Ain Shams University Maternity Hospital, Gynaecologic Outpatient Clinic from October, 2012-February, 2014. All women gave their informed consent to participate in the study, which was approved by the Research Ethics Committee of Ain Shams University, Faculty of Medicine. All participants in the present study were planned for surgical intervention for removal or exploration of an ovarian mass. None of them was pregnant or has malignant tumor other than ovarian tumor.

Five milliliter Fasting blood samples were collected from all the participants, To obtain and clarify serum, samples were left to stand at room temperature for at least 30 min to allow the blood to clot and then centrifuged at 2000 rpm for 15 min and aliquoted to be analyzed according to manufacturer's protocols of Abcam Cancer Antigen CA125 Human ELISA Kit (ab108653) supplied from Abcam Incorporation USA, that used for the measurement of human CA125, The minimum detectable concentration of CA125 by this assay is estimated to be 5 U mL^{-1}. The samples that exceeded the reading of highest standard were further diluted 2 times, absorbance value was read at 450 nm within 15 min.

Studied individuals were classified into two main groups

- **Group A:** Twenty five cases with malignant ovarian lesions (mean age 52.71±14.16) 80% were epithelial and 20% were stromal tumors, 20% were low Grade (Gx and G1) and 80% were high grade(G2 and G3), 52% were early stage (I and II) and 48% were late stage (III)

- **Group B:** Age matched 25 cases with benign ovarian lesions as a control group (mean age 42.5±11.63)

All patients of the study were subjected to complete detailed history taking, general and local examination, radio-diagnostic investigations as pelvic ultrasonography (us) and all patients were subjected to surgery for excision of the tumor mass. Then, tumor samples were sent for pathological staging and grading according to (TNM) classification. Clinical staging of the disease was done according to TNM classification (AJCC., 2010).

Tissue samples: Human ovarian tumor tissue samples (both benign and malignant) were obtained directly at the operating theater in a Petri dish on ice. These were selected to be representative of the tumor. Blood was washed by ice cold saline. The fat, necrotic tissue, skin and muscle tissue were rapidly dissected from tissue of interest. The tissue samples were wrapped in aluminum foil and immediately were chilled on ice for further RNA extraction.

RNA extraction: The RNA extraction of all samples was done by TRIzol® Reagent manufactured by Life Technologies Corporation, Carlsbad, California, which was based on a modified salt precipitation procedure in the presence of highly effective RNase inhibitors and was kept at -80°C till its use for q-Real Time PCR of HE4 and β-actin as a house keeping gene for both tissue samples.

RNA quality and quantity in µg µL^{-1}: It was then determined using an Ultraspec 1000, UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

Reverse transcription: Reverse transcription was performed using Quanti Tect® Reverse Transcription kit manufactured by (QIAGEN, Germany). It was used forcDNA synthesis with integrated removal of genomic DNA contamination, for use in real-time two-step RT-PCR.

Relative quantitative real time PCR (q-real time PCR): The volume of the first-strand reaction was brought to 20 µL with RNAase free water and template cDNA (1 µg/reaction) were amplified on an iCycler (Bio-Rad) using 10 µL 2x QuantiTect SYBR Green PCR Master Mix and 2 µL of the gene-specific oligonucleotide primers. All PCRs were done by initial activation step at 95°C for 15 min followed by 45 cycles of 15, 30 and 30 sec at 95, 50 and 72°C, respectively. Bio-Rad software was used to calculate threshold cycle (Ct) values for all target genes and for the reference gene β-actin. The expression values for the tumor samples are presented as fold expression in relation to the control sample, the actual values were calculated using the $2^{-\Delta\Delta Ct}$ equation: Then calculation of the relative quantification (RQ) or fold change is done by the following equation:

$$\text{Relative quantity (RQ)} = 2^{-\Delta\Delta CT}$$

where, ΔΔCT = [Ct HE4-Ct β actin] (malignant sample)-[Ct HE4-Ct β actin] (control sample).

Following primer sequence were used: Homo sapiens WAP four-disulfide core domain 2 (WFDC2) (>NM_006103.3) sense primer: CGGCTTCACCCTAGTCTCAG and antisense primer: CCTCCTTATCATTGGGCAGA (Richardson *et al.*, 2001), while Homo sapiens β-actin (>XM_005249820.1) sense primer: CTACGTCGCCCTGGACTTCGAGC and antisense primer: GATGGAGCCGCCGATCCACACGG (Wobser *et al.*, 2009; Mansour *et al.*, 2012).

Statistical analysis: The data was expressed as median and independent samples Mann-Whitney or Kruskall Wallis Test. Spearman's rho correlation was used to explore the relationship between HE4, CA 125 and RMI among the groups of the study. The threshold value for optimal sensitivity and specificity of HE4, CA 125 and RMI were determined by Receiver Operating Characteristics (ROC) curve. The cutoff value that maximized the sum of sensitivity and specificity was chosen for discrimination between benign and malignant groups. All statistical analysis were performed using the software package SPSS for Windows, version 15.0 (SPSS Inc., Chicago, Illinois). Significant p-value considered when it is ≤0.05.

RESULTS

Concerning the comparison between malignant and benign groups as regards the demographic data and clinical characteristics, there was no statistical significant difference between the two groups (p>0.05). Histopathological findings of the malignant group were analyzed, Epithelial Ovarian Cancer (EOC) were 20 samples (80%) and other types where 5 samples (20%), the low grade EOC samples were 4 (20%) and high grades where 16 (80%) early stages EOC were 7 samples (35%) and late stage 13 (65%) there was no statistical association between the grade and stages of the cancer group data not shown.

Table 1 shows no significant difference between the expression of HE4 in relation to the different clinicopathological factors.

The HE4 expression positivity rate and semiquantitative RT-PCR analysis in both groups of the study is shown in Table 2.

Quantitative real time PCR for HE4 is shown in Fig. 1. The best cutoff point was calculated by ROC curveto discriminate the malignant and benign cases. Best cut off point was 0.053 for HE4, 8.85 U mL^{-1} for CA 125 and 17.7 for RMI. As regards HE4 positivity rate, HE4 mRNA was>cutoff

Table 1: HE4 relationship to different risk factors in ovarian cancer group

| Clinico pathological factors | HE4 | | Total | χ^2(p) |
	Positive ≥0.053	Negative <0.053		
Age				
≥50 years	16 (100%)	0 (0%)	16	1.94 (0.164)
<50 years	7 (77.8%)	2 (22.2%)	9	
Parity				
Nulliparous	9 (81.8%)	2 (18.2%)	11	1.44 (0.231)
Multipara	14 (100%)	0 (0%)	14	
Breast feeding				
Positive	9 (100%)	0 (0%)	9	0.598 (0.439)
Negative	14 (87.5%)	2 (12.5%)	16	
Menopausal state				
Premenopausal = 1	9 (81.8%)	2 (18.2%)	11	1.44 (0.231)
Postmenopausal = 3	14 (100%)	0 (0%)	14	
Familiy history				
Positive	5 (100%)	0 (0%)	5	0.294 (0.588)
Negative	18 (90 %)	2 (10%)	20	
Smoking				
Smoker	4 (100%)	0 (0%)	4	0.808 (0.668)
Non smoker	7 (100%)	0 (0%)	7	
Passive smoker	12 (85.7%)	2 (14.3%)	14	
OCT				
Past administration	4 (100%)	0 (0%)	4	0.179 (0.672)
Never	19 (90.5%)	2 (9.5%)	21	
Total	23 (92.9%)	2 (7.1%)	25	

M.S: Menopausal state, FH: Family history, OCT: Oral contraception, US: Ultrasound score, **: p-value ≤0.01 is highly significant
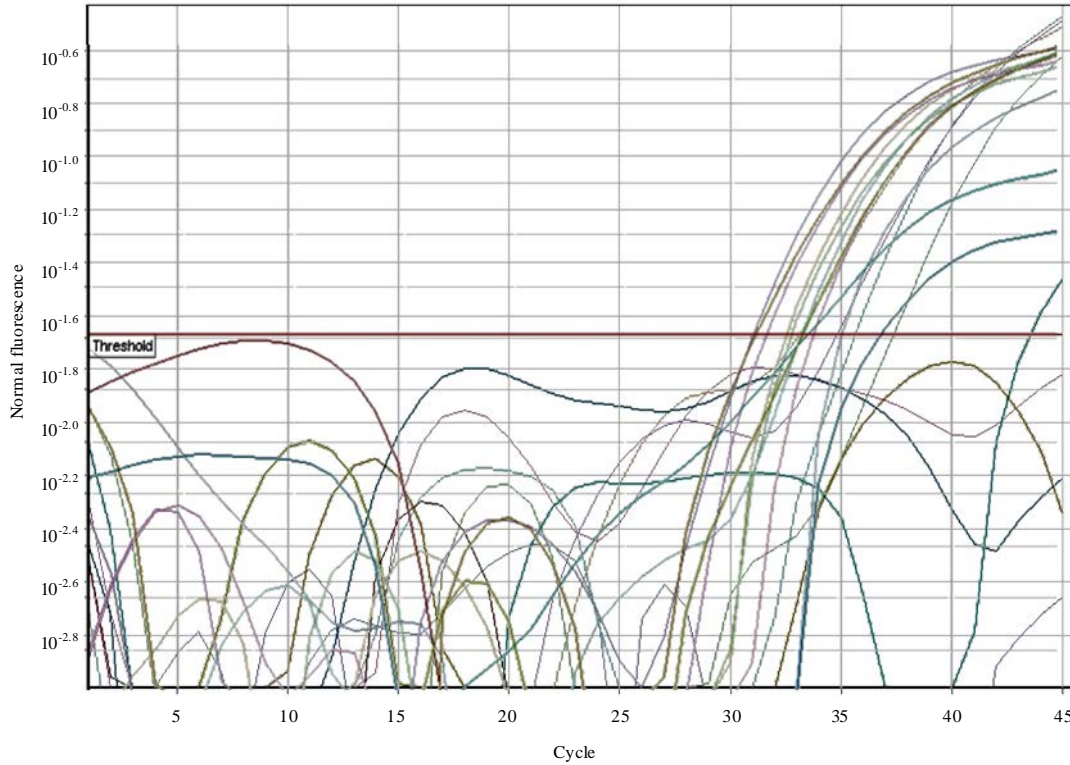
Fig. 1: Amplification curves of HE4 with its housekeeping genes by q-real time PCR in different
Groups of the study: Collective Amplification Curves of the q-real time PCR products of HE4
Gene with its housekeeping gene (β actin) in both Malignant and Benign Groups. The
transverse line indicate the threshold level (0.02)

Table 2: HE4 expression positivity rate and semiquantitative RT-PCR analysis in both groups of the study

Positivity rate (number of cases > cutoff value)	Malignant (n = 25)	Benign (n = 25)	χ^2(p)
Positive (≥0.053)	23 (92%)	1 (4%)	22.50
(0.00**)			
Negative (<0.053)	2 (16%)	24 (88%)	
Median value of different parameters in all studied groups			
HE4 expression by Q RT PCR	387.09	0.016	20.17 (0.00**)
CA125 serum levels (U mL^{-1})	22.35	0	22.34 (0.00**)
Risk of malignancy index	64.95	0	24.13 (0.00**)

**p≤0.01 is highly significant

value in 92% (23/25) of the malignant group and in 8% (2/25) of the benign group with highly
significant difference between the two groups (p<0.001). The semi-quantitative RT-PCR analysis
for HE4 in Early stage and Low grade versus control group of the study, it shows high significant
differences between the two groups and the benign cases (p<0.001 and <0.05, respectively) as
shown in Table 3.

Expression of HE4 in Ovarian tissue samples from Malignant group as measured by q-real time
PCR showed 92% sensitivity, 96% specificity and after its combination with CA 125 the sensitivity
reached to absolute value with the same accuracy and PPV. The combination of HE4 with the RMI
does not increase the significance of the RMI as shown in Table 4.

Table 3: Semi-quantitative RT-PCR analysis of HE4 in early stage and LOW grade versus control group of the study

Parameters	Semi-quantitative RT-PCR Of HE4	χ^2(P)
Benign		
Mean rank	8.063	14.96 (0.00**)
Median	0.016	
Early stage		
Mean rank	19.043	
Median	0.646	
Low grade		
Mean rank	17.033	6.07 (0.014**)
Median	0.107	

**p$\leq$0.01 is highly significant between the benign group and the studied malignant group

Table 4: Valuable combined sensitivity, specificity, accuracy, positive predictive value and negative predictive value for HE4 (at 0.053) and CA125 (at 8.85 U mL^{-1}) and RMI (at 17.7) in malignant versus benign group

Parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPP (%)	Accuracy (%)
CA125	92	100	100.0	92.6	96
HE4	92	96	95.6	96.0	94
RMI	100	96	100.0	92.3	98
Combined HE4 and CA125	100	92	100.0	92.0	96
Combined HE4 and RMI	100	92	92.6	100.0	96

Table 5: Valuable correlations between ovarian HE4, CA 125 and RMI in both groups of the study

Spearman's rho	RMI	CA 125 (U mL^{-1})
HE4 q-Real time PCR value		
Correlation coefficient	0.598	0.746
Significance	0.00**	0.00**
No.	50	50

Correlation coefficient (R) calculated by pearson's test, *p$\leq$0.05 = significant, **p$\leq$0.01 = highly significant

The expression of ovarian HE4 was positively correlated with CA 125 and RMI with high significance (p<0.01 for both) as shown in Table 5.

There is increased tissue expression Of HE4 in late stages (median = 770.69, mean rank 32.2) more that early stages (median = 0.646, mean rank 24.79) but it does not reach to statistical significance p = 0.088.

DISCUSSION

Ovarian cancer represents a heterogeneous group of distinct tumors exhibiting a wide range of morphological characteristics, clinical manifestations, genetic alterations and tumors behaviours (Conic *et al.*, 2011). Goff (2012), reviewed that ovarian cancer had always been called "The silent killer" because symptoms are not thought to develop until advanced stages, when chances of cure were very poor. In these cases, the five year survival rate is less than 40%. In contrast, the five year survival rate for tumors diagnosed at early stages, FIGO Stages I to IIA is more than 80% (Heintz *et al.*, 2006).

Search is ongoing since many years for a novel, more sensitive and more specific tumor marker or diagnostic algorithm to serve in the stratification of patients with a pelvic mass and for screening in ovarian cancer.

Several biomarkers have been examined to find alternative or additive markers that can distinguish between a benign pelvic mass and OC. Currently human epididymis protein 4 (HE4) seems to be a promising biomarker of OC (Hellstrom *et al.*, 2003; Moore *et al.*, 2008). The HE4 is a glycoprotein, over expressed by EOC. High concentration have been detected in serum from OC patients, especially patients with serous and endometrioid adenocarcinoma (Bouchard *et al.*, 2006; Drapkin *et al.*, 2005; Van *et al.*, 2011). Expression of HE4 in normal tissue is low, higher in non-ovarian cancer tissue and with the highest expression found in OC tissue (Galgano *et al.*, 2006).
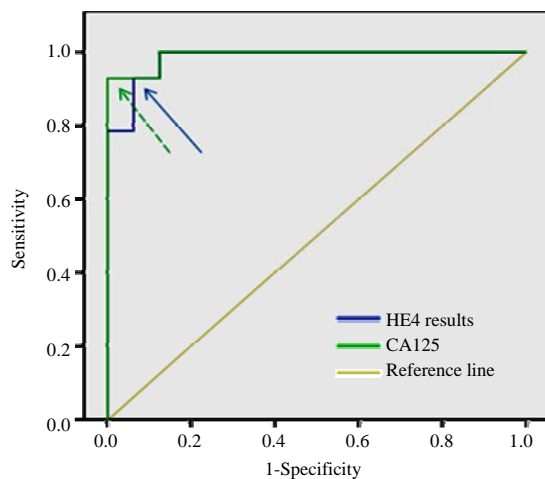
Fig. 2: Combined ROC curve analysis for, serum CA125 (U mL^{-1}) and q-RT-PCR for HE4 gene expression, in ovarian malignant group versus Ovarian benign group. In CA125 curve the area under the curve was 0.98, standard error was 0.018 and confidence limits were 0.945-1.016, Arrow denotes cut off point at 8.85 U mL^{-1} at which CA125 sensitivity was 92% and specificity was 100%, In HE4 curve the Area under the curve was 0.982, standard error was 0.018 and confidence limits were 0.946-1.018. Arrow denotes cut off point at 0.053 at which HE4 sensitivity was 92% and specificity was 96%

So HE4 is a recently proposed biomarker for ovarian cancer, serum human epididymis protein 4 (HE4) shows the highest potential for clinical use, especially as tumor marker in Epithelial Ovarian Cancer (EOC). The HE4 level is a useful preoperative test for predicting the benign or malignant nature of pelvic masses (Macedo *et al*., 2014). The HE4 is one of the newer and exceptionally useful tumor markers used in preoperative diagnostics for patients with ovarian cancer (Hellstrom *et al*., 2003; Plotti *et al*., 2012). Despite numerous studies on its diagnostic applicability, its biological role has not been fully explained (Chudecka-Glaz *et al*., 2015).

The present study extended those findings by investigating HE4 expression in human ovarian tumor tissues by quantitative real-time RT-PCR analysis, with combination between serum CA 125 and RMI with HE4 gene expression in ovarian malignancies that may be used as diagnostic tool and therapeutic target to defeat cancer cells (Fig. 2).

In this study, there was no significant differences were found between HE4 quantity and any of the studied clinicopathological factors (p>0.05) indicating that this protein is a good marker as it is not affected by any of the pathological factors. There was a highly significant difference between malignant andbenign groups as regards expression of HE4 gene by q-Real Time PCR (p<0.001) (Fig. 3). This result is consistent with the study by Macedo *et al*. (2014) which found that HE4 level is a useful preoperative test for predicting the benign or malignant nature of pelvic masses, Karlsen *et al*. (2012) which found a prominent up-regulation of HE4 expression was seen in epithelial ovarian cancer tissue, especially in serous and endometrioid adenocarcinoma. No expression wasdetected in normal ovarian tissue and a lower expressionwas observed in both benign and borderline ovariantumors compared with protein expression levels in epithelial ovarian cancer, Fujiwara *et al*. (2015) which found the median serum levels of CA 125 and HE4 were significantly higher in patients with type I and type II EOC than in patients with benign diseases
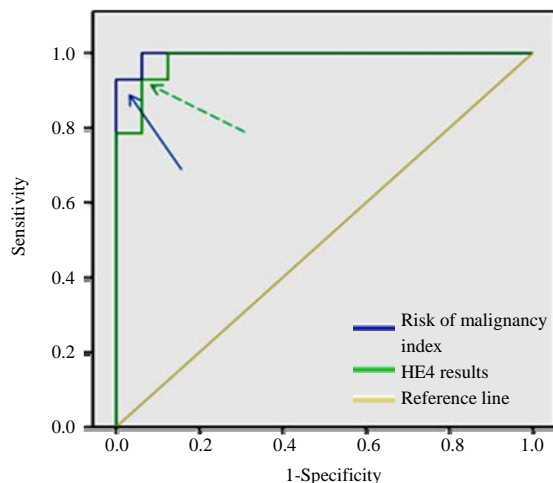
Fig. 3: Combined ROC curve analysis for, RMI and q RT-PCR for HE4 gene expression, in ovarian malignant group versus Ovarian benign group. In RMI curve the area under the curve was 0.996, standard error was 0.007 and confidence limits were 0.981-1.010. Arrow denotes cut off point at 17.7 at which RMI sensitivity was 100% and specificity was 94%. In HE4 curve the Area under the curve was 0.982, standard error was 0.018 and confidence limits were 0.946-1.018. Arrow denotes cut off point at 0.053 at which HE4 sensitivity was 92% and specificity was 96%

and in healthy volunteers, Yang *et al.* (2013) reported that the concentration of HE4 in ovarian cancer patients was significantly higher than that in benign ovarian tumour and normal control patients (p<0.01) and no statistically significant differences were observed (p>0.05) between the benign ovarian tumour lesion and normal control groups, also it is consistent with those of Moore *et al.* (2009), who detected the levels of serum HE4 in epithelial ovarian cancer (129 cases) and benign ovarian tumor patients (352 cases) and observed that HE4 was significantly increased in the epithelial ovarian cancer patients, Moore *et al.* (2008), who observed that HE4 was a useful single marker for differentiating between benign ovarian tumor and ovarian cancer patients, Galgano *et al.* (2006) which has been demonstrated that HE4 mRNA is highly expressed in ovarian cancer tissue and not expressed in benign ovarian tissue and by Wang *et al.* (1999) which studied the expression of HE4 in various ovarian tissues and revealed that HE4 was highly expressed in cancer tissue but not in normal ovarian tissue and precancerous tissues. This result showed no consistency with Chudecka-Glaz *et al.* (2015), who found that there was no difference in HE4 levels between the two study groups.

Using (0.053) as a cut off value for HE4 Gene Expression measured by real time PCR HE4 sensitivity was 92% and specificity was 96%. This is consistent with the study by Montagnana *et al.* (2009) which reported that both the sensitivity and specificity of HE4 for epithelial ovarian cancer are 98 and 100%, respectively.

In the present study, the CA 125 showed the same sensitivity of HE4 (92%), this result is consistent with the study by Karlsen *et al.* (2012) which found the sensitivity of CA 125 and HE4 91.7 and 91.3, respectively.

With respect to the overall performance as evaluated by area under the ROC curve (AUC), the RMI had the highest performance (AUC = 0.996), He4 with (AUC = 0.982) andCA 125 (AUC = 0.98)

However, this shows consistency with previous studies, as the study by Fujiwara *et al.* (2015), who found that the AUCs for HE4 were better than the AUC for CA 125 in distinguishing between benign diseases and EOC, the study by Hamed *et al.* (2013) AUC values were (0.96) for HE4 and (0.82) for (CA 125), the study by Karlsen *et al.* (2012) AUC was highest for RMI (0.905) followed by HE4 (0.864) and CA125 (0.854), respectively and for the Montagnana *et al.* (2009) study, AUC for HE4 is higher than CA 125.

There was a highly significance (r = 0.746, p<0.001) correlation between levels of CA 125 and tissue HE4 measured by real time PCR in all groups of the study. In study by Hamed *et al.* (2013) a positive correlation between serum levels of HE4 and CA 125 was observed in women with epithelial ovarian cancer, benign gynaecological disease group and control group (r = 0.5, p<0.01) which was consistent with the present study results.

There was a highly significant (r = 0.598, p<0.001) correlation between levels of RMI and HE4 measured by real time PCR in all groups of the study.

In the current study HE4 was highly expressed in early stages and low grades ovarian tumours in comparison to the benign cases (p = 0.001and 0.014, respectively). This suggests the possibility of usage this gene as an early diagnostic marker in ovarian cancer which is a novel finding in this study. As the study done by Fujiwara *et al.* (2015) compared between results of 2 types in cancer group only, type 1 and 2 (low and high grades) EOC.

In the present study, using ROC curves and AUCs, it was found the HE4, RMI and CA 125 to be closely accurate for stratification of ovarian cancers and by combination of HE4 with CA 125 or RMI will result in absolute sensitivity (100%).

We observed that increased HE4 tissue expression in the ovarian cancer are associated with a poor prognosis with higher disease stages but it does not reach to statistical significance (p>0.05), this is supported by a study done by Jiang *et al.* (2013) which investigated HE4 in the endometrial carcinoma.

CONCLUSION

For conclusion, HE4 could be a good prognostic marker of ovarian cancer, which will have a therapeutic impact on these patients.

ACKNOWLEDGMENT

REFERENCES

AJCC., 2010. Cancer Staging Manual. 7th Edn., American Joint Committee on Cancer, New York, USA.

Anastasi, E., G.G. Marchei, V. Viggiani, G. Gennarini, L. Frati and M.G. Reale, 2010. HE4: A new potential early biomarker for the recurrence of ovarian cancer. Tumor Biol., 31: 113-119.

Bouchard, D., D. Morisset, Y. Bourbonnais and G.M. Tremblay, 2006. Proteins with whey-acidic-protein motifs and cancer. Lancet Oncol., 7: 167-174.

Cannistra, S.A., 2004. Cancer of the ovary. N. Engl. J. Med., 351: 2519-2529.

Chudecka-Glaz, A.M., A.A. Cymbaluk-Ploska, J.L. Menkiszak, E. Pius-Sadowska, B.B. Machalinski, A. Sompolska-Rzechula and I.A. Rzepka-Gorska, 2015. Assessment of selected cytokines, proteins and growth factors in the peritoneal fluid of patients with ovarian cancer and benign gynecological conditions. Onco Targets Ther., 23: 471-485.

Conic, I., I. Dimov, D. Tasic-Dimov, B. Djordjevic and V. Stefanovic, 2011. Ovarian epithelial cancer stem cells. Sci. World J., 11: 1243-1269.

Drapkin, R., H.H. von Horsten, Y. Lin, S.C. Mok, C.P. Crum, W.R. Welch and J.L. Hecht, 2005. Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. Cancer Res., 65: 2162-2169.

Ferlay, J., H.R. Shin, F. Bray, D. Forman, C. Mathers and D.M. Parkin, 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer, 127: 2893-2917.

Fujiwara, H., M. Suzuki, N. Takeshima, K. Takizawa and E. Kimura *et al.*, 2015. Evaluation of human epididymis protein 4 (HE4) and Risk of Ovarian Malignancy Algorithm (ROMA) as diagnostic tools of type I and type II epithelial ovarian cancer in Japanese women. Tumor Biol., 36: 1045-1053.

Galgano, M.T., G.M. Hampton and H.F. Frierson Jr., 2006. Comprehensive analysis of HE4 expression in normal and malignant human tissues. Mod. Pathol., 19: 847-853.

Goff, B., 2012. Symptoms associated with ovarian cancer. Clin. Obstet. Gynecol., 55: 36-42.

Hamed, E.O., H. Ahmed, O.B. Sedeek, A.M. Mohammed, A.A. Abd-Alla and H.M.A. Ghaffar, 2013. Significance of HE4 estimation in comparison with CA125 in diagnosis of ovarian cancer and assessment of treatment response. Diagn Pathol., Vol. 8.

Heintz, A.P.M., F. Odicino, P. Maisonneuve, M.A. Quinn and J.L. Benedet *et al.*, 2006. Carcinoma of the ovary. Int. J. Gynaecol. Obstet., 95: 161-192.

Hellstrom, I., J. Raycraft, M. Hayden-Ledbetter, J.A. Ledbetter and M. Schummer *et al.*, 2003. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. Cancer Res., 63: 3695-3700.

Holschneider, C.H. and J.S. Berek, 2000. Ovarian cancer: Epidemiology, biology and prognostic factors. Seminars Surg. Oncol., 19: 3-10.

Huhtinen, K., P. Suvitie, J. Hiissa, J. Junnila and J. Huvila *et al.*, 2009. Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts. Br. J. Cancer, 100: 1315-1319.

Jacobs, I., D. Oram, J. Fairbanks, J. Turner, C. Frost and J.G. Grudzinskas, 1990. A risk of malignancy index incorporating CA 125, ultrasound and menopausal status for the accurate preoperative diagnosis of ovarian cancer. Br. J. Obstet. Gynaecol., 97: 922-929.

Jiang, S.W., H. Chen, S. Dowdy, A. Fu and J. Attewell *et al.*, 2013. HE4 transcription-and splice variants-specific expression in endometrial cancer and correlation with patient survival. Int. J. Mol. Sci., 14: 22655-22677.

Karlsen, M.A., N. Sandhu, C. Hogdall, I.J. Christensen and L. Nedergaard *et al.*, 2012. Evaluation of HE4, CA125, Risk of Ovarian Malignancy Algorithm (ROMA) and risk of malignancy index (RMI) as diagnostic tools of epithelial ovarian cancer in patients with a pelvic mass. Gynecol. Oncol., 127: 379-383.

Kobel, M., S.E. Kalloger, N. Boyd, S. McKinney and E. Mehl *et al.*, 2008. Ovarian carcinoma subtypes are different diseases: Implications for biomarker studies. PLoS Med., Vol. 5. 10.1371/journal.pmed.0050232

Li, J., H. Chen, A. Mariani, D. Chen and E. Klatt *et al.*, 2013. HE4 (WFDC2) promotes tumor growth in endometrial cancer cell lines. Int. J. Mol. Sci., 14: 6026-6043.

Lu, K.H., S. Skates, M.A. Hernandez, D. Bedi and T. Bevers *et al.*, 2013. A 2-stage ovarian cancer screening strategy using the Risk of Ovarian Cancer Algorithm (ROCA) identifies early-stage incident cancers and demonstrates high positive predictive value. Cancer, 119: 3454-3461.

Macedo, A.C.L., M.I. da Rosa, S. Lumertz and L.R. Medeiros, 2014. Accuracy of serum human epididymis protein 4 in ovarian cancer diagnosis: A systematic review and meta-analysis. Int. J. Gynecol. Cancer, 24: 1222-1231.

Mansour, A., M. Nabil, R. Ali-Labib, H. Said and F. Annos, 2012. Reciprocal expression of survivin and SMAC/DIABLO in primary breast cancer. Med. Oncol., 29: 2535-2542.

Montagnana, M., G. Lippi, E. Danese, M. Franchi and G.C. Guidi, 2009. Usefulness of serum HE4 in endometriotic cysts. Br. J. Cancer, 101: 548-548.

Moore, R.G., A.K. Brown, M.C. Miller, S. Skates and W.J. Allard *et al.*, 2008. The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass. Gynecol. Oncol., 108: 402-408.

Moore, R.G., D.S. McMeekin, A.K. Brown, P. DiSilvestro and M.C. Miller *et al.*, 2009. A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. Gynecol. Oncol., 112: 40-46.

Plotti, F., S. Capriglione, C. Terranova, R. Montera and A. Aloisi *et al.*, 2012. Does HE4 have a role as biomarker in the recurrence of ovarian cancer? Tumor Biol., 33: 2117-2123.

Richardson, R.T., P. Sivashanmugam, S.H. Hall, K.G. Hamil and P.A. Moore *et al.*, 2001. Cloning and sequencing of human *Eppin*: A novel family of protease inhibitors expressed in the epididymis and testis. Gene, 270: 93-102.

Salani, R., A. Santillan, M.L. Zahurak, R.L. Giuntoli, G.J. Gardner, D.K. Armstrong and R.E. Bristow, 2007. Secondary cytoreductive surgery for localized, recurrent epithelial ovarian cancer. Cancer, 109: 685-691.

Scholler, N., M. Crawford, A. Sato, C.W. Drescher and K.C. O'Briant *et al.*, 2006. Bead-based ELISA for validation of ovarian cancer early detection markers. Clin. Cancer Res., 12: 2117-2124.

Siegel, R., D. Naishadham and A. Jemal, 2013. Cancer statistics. CA: Cancer J. Clin., 63: 11-30.

Van Dekken, H., J.C. Alers, P.H.J. Riegman, C. Rosenberg, H.W. Tilanus and K. Vissers, 2001. Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. Am. J. Pathol., 158: 1961-1967.

Van, G.T., I. Cadron, E. Despierre, A. Daemen and K. Leunen *et al.*, 2011. HE4 and CA125 as a diagnostic test in ovarian cancer: Prospective validation of the risk of ovarian malignancy algorithm. Br. J. Cancer, 104: 863-870.

Wang, K., L. Gan, E. Jeffery, M. Gayle and A.M. Gown *et al.*, 1999. Monitoring gene expression profile changes in ovarian carcinomas using cDNA microarray. Gene, 229: 101-108.

Winstead, E.R., 2009. Ovarian cancer markers validated for early detection. Cancer Bulletin No. 6, National Cancer Institute, USA.

Wobser, H., C. Dorn, T.S. Weiss, T. Amann and C. Bollheimer *et al.*, 2009. Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells. Cell Res., 19: 996-1005.

Yang, Z., Z. Luo, B. Zhao, W. Zhang and J. Zhang *et al.*, 2013. Diagnosis and preoperative predictive value of serum HE4 concentrations for optimal debulking in epithelial ovarian cancer. Oncol. Lett., 6: 28-34.