RESEARCH ARTICLE

Human Epididymis Protein 4 Reference Intervals in a Multiethnic Asian Women Population

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Abstract

    <u>Background</u>: Ovarian cancer is ranked as the fifth most common cause of cancer death in women. In Malaysia, it is the fourth most common cancer in females. CA125 has been the tumor marker of choice in ovarian cancer but its diagnostic specificity in early stages is only 50%. Hence, there is a critical need to identify an alternative tumor marker that is capable of detecting detect ovarian cancer at an early stage. HE4 is a new tumor marker proposed for the early diagnosis of ovarian cancer and disease recurrence. Currently, none of the normal ranges of HE4 quoted in the literature are based on data for a multiethnic Asian population. Therefore, the aim of this study was to determine reference intervals for HE4 in an Asian population presenting in University Malaya Medical Centre, a tertiary reference hospital. <u>Materials and Methods</u>: 300 healthy women were recruited comprising 150 premenopausal and 150 postmenopausal women, aged from 20-76 years. All women were subjected to a pelvic ultrasonograph and were confirmed to be free from ovarian pathology on recruitment. Serum HE4 levels were determined by chemiluminescent microparticle immunoassay (CMIA, Abbott Architect). The reference intervals were determined following CLSI guidelines (C28-A2) using a non-parametric method. <u>Results</u>: The upper limits of the 95th percentile reference interval (90%CI) for all the women collectively were 64.6 pmol/L, and 58.4 pmol/L for premenopausal) and 69.0 pmol/L for postmenopausal. The concentration of HE4 was noted to increase with age especially in women who were more than 50 years old. We also noted that our proposed reference limit was lower compared to the level given by manufacturer Abbott Architect HE4 kit insert (58.4 vs 70 pmol/L for premenopausal group and 69.0 vs 140 pmol/L in the postmenopausal group). The study also showed a significant difference in HE4 concentrations between ethnic groups (Malays and Indians). The levels of HE4 in Indians appeared higher than in Malays (p<0.05), while no significant differences were noted between the Malays and Chinese ethnic groups. <u>Conclusions</u>: More data are needed to establish a reference interval that will better represent the multiethnic Malaysian population. Probably a larger sampling size of equal representation of the Malay, Chinese, Indians as well as the other native ethnic communities will give us a greater confidence on whether genetics plays a role in reference interval determination.

Keywords: Ovarian cancer - biomarker - HE4 - Asian women - ethnic groups

Introduction

    Ovarian cancer is a silent disease which presents with vague symptoms such as pelvic or abdominal pain, urinary frequency and or urgency and increased abdominal size or bloating. Majority of these patients can present with adnexal mass, with or without evidence of metastatic disease (Moore et al., 2008). In most cases ovarian cancer is diagnosed in advanced stages (FIGO III-IV) which gives the median survival of 18-24 months with a 80% probability of disease recurrence within 5 years (Anastasi et al., 2010). The survival rate at this advanced stage is <20% despite aggressive surgery and chemotherapy (Hellstrom et al., 2003). Ovarian cancer accounts for nearly 4% of all cancers among women and has been ranked as the fifth most common cause of cancer death in women (Escudero et al., 2011). In Malaysia, ovarian cancer was ranked as the fourth most common cancer in females in 2007 (Malaysian Cancer Statistics, 2007).

    According to NACB (National Academy of Clinical Biochemistry) tumor markers act as surrogate indicators that increase or decrease the clinician's suspicion in events concerning cancer onset, recurrence or progression that will lead to specific treatment modalities to decrease the risk of such events (Sturgeon and Duffy, 2008). Cancer antigen 125 (CA125) has been the tumor marker of choice for ovarian cancer. However, the diagnostic sensitivity is low when it is related to tumor stage, (abnormal levels were seen in 50% of stage 1 patients and 80-90% in stage III-IV) (Escudero et al., 2011) and 20% of epithelial ovarian cancers do not expressed CA125 (Bast and Badgwell, 2005).

    High concentrations of CA125 can also be found in malignancies of non-ovarian origin (endometrium and endocervix), other epithelial tumours (lung cancer) and nonepithelial malignancies (lymphoma). Abnormal

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concentrations of CA125 were also noted in benign disease cases such as effusions, liver or renal failure and also in benign gynecologic conditions such as ovarian cyst, myomas and endometriosis (Niloff et al., 1984; Fuith and Daxenbichler, 1987; Meden and Fattahi-Meibodi, 1998; Buamah, 2000). Due to its limited diagnostic performance, CA125 is not recommended for regular screening or diagnosis. However, it is recommended when used in combination with transvaginal ultrasound for early detection of ovarian cancer only in high risk women and for the differential diagnosis of suspicious pelvic masses in postmenopausal women. It is also recommended for monitoring treatment, prognosis and disease relapse in known ovarian cancer patients. The search for a new tumor marker is needed where it can complement CA125 or be a better discriminator between malignant and benign gynecological disease particularly in the cases of ovarian cancer.

HE4 is a new tumor marker proposed for ovarian cancer. The HE4 gene also known as WFDC2 (WAP four-disulfide core domain 2) is noted to be highly expressed in ovarian cancer but not in normal tissue.

This gene belongs to a four-disulphide core family which is composed of a group of heat and acid stable proteins with different functions which includes protease inhibitor (Anastasi et al., 2010; Montagnana et al., 2011). HE4 was initially discovered in the epithelium of the distal epididymis and was thought to be a protease inhibitor in sperm maturation. It is expressed in the early stages of ovarian cancer and also acts as an early indicator of disease recurrence (Hellstrom and Hellstrom, 2008). Studies also showed that HE4 expression is subtype specific being mostly limited to serous and endometroid subclasses of epithelial ovarian cancer (Bingle and Singleton, 2002).

Measurement of HE4 in serum of patients with ovarian carcinoma was first attempted by Hellstrom and colleagues in 2003. They found that HE4 was less positive in non malignant disease and can be more useful than CA 125 (Kirchhoff, 1998). HE4 gave 80% sensitivity in diagnosing late stage cases and 95% specificity. As a single marker, HE4 has a highest sensitivity of 83% in detection of Stage 1 disease and patients with pelvic mass. HE4 is not influenced by menopausal status as compared to CA125. In contrast to CA125, other studies showed that mean serum HE4 concentration was significantly increased in patients with both endometrial and ovarian cancer but not with ovarian endometriomas or other types of endometriosis (Bingle and Singleton, 2002). Moore and colleagues showed that when HE4 is used in combination with CA125, the sensitivity is higher compared to using either tumor markers alone. This combination might be the most effective tool in the stratification of women at high and low risk for epithelial ovarian cancer (sensitivity -92% - in post menopausal women and 76% in premenopausal women) at the specificity of 75%. This finding has been the centre of the predictive algorithm (Risk of Ovarian Malignancy Algorithm – ROMA) for assessing Epithelial Ovarian Cancer risk in women presenting with pelvic mass (Moore et al., 2009). None of the normal ranges of HE4 quoted in the literature were conducted in multiethnic Asian population. In this study, we aimed to evaluate the normal ranges of HE4 in the multiethnic population of Malaysia.

Materials and Methods

Patient population and study design

This cross-sectional study involved recruitment of three hundreds healthy female participants from the Outpatient Department and Menopausal Clinic of the Obstetrics and Gynaecology Department University of Malaya Medical Centre, (UMMC). Socio-demographic variables collected were age and menopausal status. The participants were divided into two groups comprising one-hundred and fifty of premenopausal women (age range 20-49 years, median 35 years) with regular menstrual cycles and a second group of one-hundred and fifty of postmenopausal women (age range 47-75 years, median 59 years) with absence of menses for more than one year (physiological menopause).

Pregnancy and the presence of other neoplastic disease were considered as exclusion criteria. Pelvic ultrasonography was performed by sonography technicians in order to confirm the absence of ovarian pathologies and in cases where suspicious arose, transvaginal ultrasonography was performed by the consultant gynecologist. An informed written consent was obtained at recruitment and before any sampling from all the participants. This was approved by the University Malaya Medical Centre Medical Ethics Committee.

Serum collection

All blood samples were acquired according to a standard collection protocol. About 5mls of blood were collected in a Red Top Vacutainer (plain tube). The samples were centrifuged at 3,000 rpm for 5 minutes after being clotted for 60-90 minutes. Aliquots of serum were stored at (-80°C) until analysis were performed.

Biomarker assays

Levels of HE4 were determined using ARCHITECT HE4 assay (Abbott Diagnostics) which is a chemiluminescent microparticle immunoassay (CMIA) for quantitative determination of HE4 antigen in human serum. It is a two-step immunoassay: which involved combination of 2H5 anti-HE4 coated paramagnetic microparticles and the sample (first step). The HE4 antigen present in the sample binds to the anti-HE4 coated microparticles. After washing, a 3D8 anti-HE4 acridinium labeled conjugate is added to create a reaction mixture (second step). After the second wash cycle, trigger solutions which contain hydrogen peroxide and sodium hydroxide were added to the reaction mixture. The generated chemiluminescent reaction is measured by ARCHITECT System optics in the form of relative light units (RLU). The amount of HE4 antigen in the sample is directly related to the RLUs.

Statistical analysis

Statistical analyses in this study were performed using the SPSS (IBM SPSS version 20.0) while the determination of 95% reference interval (90%CI) were carried out using MedCalc version 12 @ 1993-2012

software. The distributions of HE4 in all the study groups (healthy premenopausal and postmenopausal women) were defined by the Kolmogorov-Smirnov test. Categorical variables were presented in frequency and percentage while numerical variables were presented in mean with standard deviation (SD), median with inter-quartile range (IQR), minimum and maximum value. Uni-variate test such as independent sample t-test was used to determine the association between independent and dependent variables. Multiple comparisons between ethnicity and concentration of HE4 levels were performed using ANOVA test. In all analyses, p- values of less than 0.05 were considered to be statistically significant.

Results

The characteristics of the study parameters includes the age, menopausal status and ethnicity. Majority of the respondents belong to the Malay group which constitutes 215 women (71.7%). The mean age of all the women is 42.8 years, with the minimum age of 20 years and the maximum is 76 years old. The median HE4 for the all women were 36.6 pmol/L, with 33.8 pmol/L and 40.3 pmol/L for pre and postmenopausal women group respectively. The minimum HE4 concentration was 17 pmol/L and maximum concentration was 75.4 pmol/L. The reference limits for HE4 in premenopausal, postmenopausal and all women groups were determine following the guidelines from CLSI (Clinical and Laboratory Standards Institute) - C28-A2 by using the upper limit of the 95th percentile reference interval with 90% confidence interval. The HE4 concentration were not normally distributed, thus a non-parametric methods were used. From Table 1, the upper 95% reference intervals for all women was 64.6 pmol/L, 58.4 pmol/L for the premenopausal and 69.0 pmol/L for the postmenopausal groups. There is a statistically significant difference between the mean of the premenopausal (35.0 pmol/L) and postmenopausal women (42.0 pmol/L), (p-value<0.001). The mean concentration for HE4 was 42.8 pmol/L for all the women (pre and
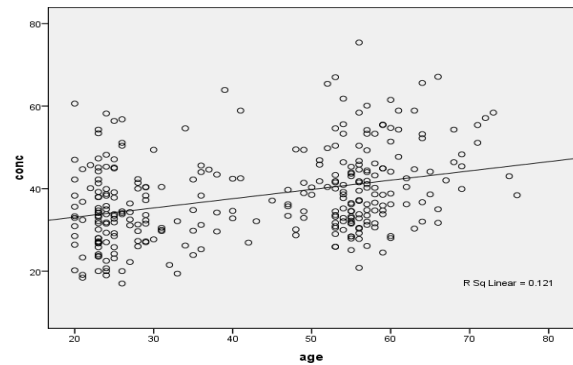
Figure 1. Scatter Plot Showing the Relation of Serum HE4 with Age

postmenopausal). From Figure 1, the scatter plot showed an association between the concentration of HE4 and age where serum HE4 clearly rise consistently with age (p<0.001). Comparison of HE4 concentrations between the ethnicities revealed that the level of HE4 was noted to be higher in Indian compared to Malays (p<0.05). There were no difference between the concentration of HE4 in Malays and Chinese. The comparison of HE4 levels between the age groups of women (Table 1) showed that the mean of the HE4 levels rises with age (p<0.001), especially in Group 4 (50-65 years) and Group 5 (>65 years). Similar findings were noted in the postmenopausal group (Group 3. >65years) (p<0.05). No significant association was seen in premenopausal participants.

Discussion

Ovarian cancer is well known for its late presentation and has been ranked as the fifth cause of cancer death from gynecological cancer. CA125 has been used as a marker for detection of epithelial ovarian cancer, which constitutes more than 90% of ovarian cancer (Montagnana et al., 2011). Limitations exist for CA125 which hampers it from being a screening tool for ovarian cancer.

HE4 (human epididymis-specific protein 4) has been identified as a potential serum marker for detection and monitoring of recurrences in ovarian cancer (Kirchhoff, 1998; Bingle and Singleton, 2002). It was initially identified in the epithelium of distal epididymis and found to be overexpressed in ovarian carcinomas. It can be used alone or in combination with CA125 in triaging women presenting with pelvic masses. It is used in ROMA (Risk of Ovarian Malignancy Algorithm) studies where the levels of HE4, CA125 and menopausal status are calculated to classify patients as being low or high risk for Epithelial Ovarian Cancer (EOC) (Moore et al., 2009). These studies have shown that 87.3% of patients with EOC and benign tumours were correctly classified as high and low risk. Negative predictive value in the population tested was 95%.

Using a combination of CA125 and transvaginal ultrasound were noted to be an effective screening tool to detect early ovarian malignancy and had even resulted in unnecessary surgical intervention (Menon and Gentry-Maharaj, 2009). HE4 has been developed and proposed as a potential serum marker for detection

Table 1. Comparison of HE4 Levels between Age Groups and Its Reference Limits in Healthy Women

	Age group (n)	Mean (SD)	95%CI	p-value
All	20-29 (110)	34.4 (9.4)	32.6-36.2	(ref)
	30-39 (25)	35.7 (10.8)	31.2-40.1	1
	40-49 (20)	37.9 (7.8)	34.2-41.6	1
	50-65 (129)	41.2 (11.2)	39.2-43.1	<0.001
	>65 (16)	50.0 (13.6)	42.8-57.3	<0.001
Premenopausal	20-39 (110)	34.4 (9.4)	32.6-36.2	(ref)
	30-39 (25)	35.7 (10.8)	31.2-40.1	1
	40-49 (15)	37.6 (8.3)	33.0-42.3	0.678
Postmenopausal	40-49 (5)	38.7 (6.9)	30.1-47.2	(ref)
	50-65 (129)	41.2 (11.2)	39.2-43.1	1
	>65 (16)	50.0 (13.6)	42.8-57.3	0.011

	ALL (n=300)	Premenopausal (n=150)	Postmenopausal (n=150)
Reference limit for serum HE4 levels in healthy women (pmol/L)			
95% reference interval	20.0-64.6	19.1-58.4	25.7-69.0
Lower limit (90%CI)	20.0 (19.0-22.2)	19.1 (17.0-20.0)	25.7 (20.8-27.9)
Upper limit (90%CI)	64.6 (58.9-75.4)	58.4 (54.3-63.9)	69.0 (61.8-89.2)

of early stages EOC (mainly FIGO stage 1 and 2) and as early marker for recurrence post treatment (Anastasi et al., 2010). Many studies have been conducted on its clinical utility and reference ranges (Bolstad and Oijordsbakken, 2012; Moore et al., 2012; Park et al., 2012). Most of them were carried out on Caucasian women with few studies involving Asian women. In this study based in University Malaya Medical Centre, Kuala Lumpur, a total of 300 healthy women with no previous gynecological or malignant disease were recruited with 150 participants belonging to the premenopausal group while the remainder were in the postmenopausal group. All three major ethnicity were involved.

Park et al. (2012) had established the reference limit for HE4 and CA125 in a 2,400 healthy South Korean women (Park et al., 2012). They showed that the reference limit of HE4 increases with age (50-65 year) but those of CA125 tend to decreased. The upper reference limit of 97.5th percentile (95%CI) were calculated based on age groups (no data regarding menopausal status), ranging from 31.8-34.0 pmol/L (20-65 years old). Our findings concurred with this study that there was a trend towards a higher reference limit in older women (Figure 1), especially noticeable between the younger (40-49 years old) and older (>65 years old) in the post-menopausal groups (p<0.05), (Table 3 and 4). There was no significant difference in the concentration of HE4 among the different age groups in the premenopausal women. We also noted that reference limits (95% reference interval with 90%CI) for HE4 in our study increases with menopausal state (58.4 pmol/L and 69.0 pmol/L for the pre and post menopausal groups respectively), but this could merely reflecting the increasing in age rather than being menopausal status per se. Similar trend were noted in studies on Caucasian women showing inclination of HE4 concentration to increase with age (Bolstad and Oijordsbakken, 2012; Moore et al., 2012). Our study also showed a significant difference in HE4 concentrations between the ethnicities (Malays and Indians). The levels of HE4 in Indian appeared higher than in Malays (p<0.05). There were no difference noted in the level of HE4 between the Malays and Chinese.

Bolstad and Oijordsbakken (2012) measured HE4 in 1,519 serum samples from donors aged 18-86 years old from both sexes in Norway. This study involved the establishment of reference limits for HE4 using 97.5% percentile with 90% confidence interval (CI) and investigating factors influencing HE4 levels in healthy subjects such as age, sex, body mass index, smoking habits and creatinine. Smokers tend to give 29% higher concentration of HE4 compared with non smokers. High BMI was noted to be associated with lower HE4 values while higher creatinine levels are associated with high HE4 values. The proposed reference limits are age-dependent, ranging from 49.1-52.6 pmol/L (18-50 years old) and 54.1-72.6 pmol/L (55-86 years old) (Bolstad and Oijordsbakken, 2012) . The difference in concentration of HE4 between sexes is relatively small. Male is associated with lower HE4 levels and the age related increase of HE4 is more noticeable in this group. In a study by Moore et al. (2012), they found that level of HE4 is lower during

pregnancy and similar trend of higher level in post menopausal women were observed (118.9 pmol/L and 167.8 pmol/L for pre and post menopausal group based on upper reference limit of the 95th percentile reference interval with 90%CI) (Moore et al., 2012).

HE4 has been noted to be expressed differently during the different phases of the menstrual cycle in healthy young women. Lower level of HE4 was observed in follicular phase compared to slightly higher level during ovulatory and luteal phases, especially in women below 35 years (Anastasi et al., 2010).

In our study, the upper reference limit is two times higher in all group (pre and postmenopausal) as compared with the South Korean study (Park et al., 2012), (64.6 vs 33.2 pmol/L). However, our data seems to concur with data from the Norway (Bolstad and Oijordsbakken, 2012), but lower than the values found by Moore (2012). We also noted that our proposed reference limit are lower compared to the level given by manufacturer Abbott Architect HE4 kit insert (58.4 vs 70 pmol/L for premenopausal group and 69.0 vs 140 pmol/L in the postmenopausal group). This could be due to genetic or differences in body mass index. This study population is overwhelmingly Malay dominant which is reflective of the racial composition in Malaysia. Probably a greater representative of the Chinese, Indians, as well as the other native ethnic communities will give us a better picture on whether genetics play a role in determining reference upper limit or normal. More data is needed to establish a better reference limit that will represent Malaysian population. In addition, our study was limited by its relatively small sample size and was also lacking correlation to CA125.

In conclusion, the clinical utility of the serum concentration of HE4 should be further enhanced by establishing appropriate reference intervals in our multiethnic Asian women. This will further enable us to determine the appropriate concentration of HE4 to diagnose EOC in order to guide further treatment and recurrence.

Acknowledgements

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