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Pair-Box (PAX8) protein-positive expression is associated with poor disease outcome in women with endometrial cancer

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BACKGROUND: The *Pax8* transcription factor genes have a role in cell differentiation and cell growth, and silencing of Pax8 in cell cultures results in cell death. The aims of this study were to determine the expression and correlation of Pax8 protein with several clinicopathological variables in patients with endometrial cancer.

METHODS: The following clinical parameters from 229 patients were used for correlation with Pax8 expression; age, histological subtype, myometrial depth of invasion, lymphovascular invasion (LVI), the International Federation of Gynecology and Obstetrics grade, lymph nodes status, and disease status.

RESULTS: A positive association of $Pax8^+$ expression was found with high tumour grade ($P = 0.002$), LVI + ($P = 0.0186$), and type II tumour subtype ($P < 0.0001$) in univariate analysis. Survival analysis showed an association of Pax8 and 5-year overall survival probability ($P = 0.01486$), 80.04% for patients with $Pax8^-$ and 55.59% for patients with $Pax8^+$. There was also an association of Pax8 and 5-year disease-free survival probability ($P = 0.02028$), 72.12% for patients with $Pax8^-$ *vs* 49.88% for patients with $Pax8^+$. Finally, an association of $Pax8^+$ and shorter recurrence-free survival was also found ($P = 0.00203$), with 74.36% for $Pax8^-$ and 52.11% for $Pax8^+$.

CONCLUSION: Overexpression of Pax8 protein by endometrial cancer is associated with poor disease outcomes. Inhibition of Pax8 may be a very attractive targeted therapy for selective patients.

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Endometrial carcinoma (EC) is the most common gynaecologic malignancy in the United States and it is the fifth leading cause of cancer-related death in women (Jemal *et al*, 2009). Endometrioid adenocarcinomas (EAC) account for more than 80% of cases and tend to present as low-grade, early-stage tumours with favourable outcomes. Meanwhile, uterine serous carcinomas (USC) represent a minority (3–10%) of total endometrial cancer cases. They are usually high-grade tumours with deep myometrial invasion (MI), lymphovascular invasion (LVI), and a more aggressive clinical course (Part, 2004; Benito *et al*, 2009; Kitchener and Trimble, 2009). Despite the validation of the prognostic value of a number of biomarkers in EC, clinicopathological factors such as tumour grade, stage, histological subtypes, LVI, and depth of MI are still considered as the most reliable prognostic parameters. High-grade tumour, advanced stage disease, presence of LVI, deep MI (outer third) and type II (USC and clear cell subtypes) endometrial adenocarcinoma constitute a high-risk group for recurrence and aggressive outcome in comparison to a low-risk group defined by low tumour grade, tumour confined to uterine corpus at presentation, absence of LVI, superficial MI (inner third), and type I (EAC and mucinous subtypes) endometrial adenocarcinoma (Figge *et al*, 1983; Keys *et al*, 2004; Part, 2004).

Pax8 is a member of the pair-box (PAX) family of transcription factor genes. It is important in the organogenesis of the thyroid, kidney, and mullerian tracts (Poleev *et al*, 1990; Mittag *et al*, 2007). Interestingly, Pax8 expression was found to be restricted to secretory cells of the fallopian tube epithelium, which most recently has been suggested as the cell of origin for serous ovarian carcinomas (Bowen *et al*, 2007). Otherwise, the interest in Pax8 in the reproductive tract has mainly focused on its use as a marker to distinguish carcinoma of gynaecologic origin *vs* carcinoma from other organs and from mesothelioma (Nonaka *et al*, 2008; Laury *et al*, 2010, 2011). The potential role of Pax8 in the endometrium has been rarely explored (Brunner *et al*, 2011). In this study, our aim is to define whether the expression of Pax8 protein in formalin-fixed samples from 229 patients correlated with disease outcome, such as recurrence rate, time to recurrence, and survival as well as with clinical–pathological factors such as tumour subtype, tumour grade and stage, LVI, depth of MI, and lymph node status.

MATERIALS AND METHODS

Patient population

After obtaining Institutional Review Board (IRB) approval, pathology archives were searched for cases of endometrial

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malignant neoplasms from January 2000 to December 2010. A chart review was conducted with extraction of clinical information, including the patients' age at the time of diagnosis, the surgical stage, the post-operative therapy, the recurrence-free survival (RFS), the disease-free survival (DFS), the site of recurrence, the cause and the time of death. All patients underwent a complete surgical staging procedure, including a total abdominal hysterectomy with bilateral salpingo-oophorectomy, pelving washing, with or without pelvic and para-aortic lymph node dissection depending on the tumour grade and the tumour subtypes. Patients were treated according to the National Comprehensive Cancer Network guidelines (www.cancer.gov).

Histological evaluation

Tumour grade was assessed using the International Federation of Gynecology and Obstetrics (FIGO) system and by nuclear grading. The FIGO grading was determined as follows; tumours with $<5\%$ solid areas were grade 1 (G1), tumours with 5–50% solid areas were grade 2 (G2) and tumours with $>50\%$ solid areas were grade 3 (G3). Tumours nuclear grade was determined by the variation in nuclear size and shape, chromatin distribution, and size of the nucleoli. Tumour stage was assigned based on 1988 FIGO surgical staging guidelines (FIGO, 1989). All slides were examined by an expert gynaecologic pathologist for confirmation of the tumour type, tumour grade, depth of MI, presence of LVI and positive lymph nodes.

Four-μm sections from formalin-fixed, paraffin-embedded tissues were processed for immunohistochemistry (IHC). Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 5 min. Antigen retrieval was carried out in high citrate buffer for 3 min in a steam-cooker. Then, sections were incubated overnight with Pax8 antibody (monoclonal antibody, 1 : 400 dilutions; Biocare lab, San Francisco, CA, USA). A subsequent reaction was performed with the biotin-free HRP enzyme-labelled polymer of the Envision plus detection system (Dakocytomation, Carpinteria, CA, USA). Diaminobenzidine complex was used as the chromogen. Normal kidney and thyroid tissue were used as positive controls. In negative controls, a normal goat serum was used instead of the primary antibody resulting in a lack of detectable staining. Two pathologists (PMF and DS) reviewed the IHC slides on a double-headed microscope; only positive nuclear staining was considered positive and the staining intensity was interpreted as negative, weak, moderate, or strong. Both pathologists were unaware of the primary diagnosis. Only the staining intensity was taken into consideration for primary statistical analysis and it was divided into two groups; negative/weak was considered as negative (group I) and moderate/strong was considered as positive (group II).

Statistical analyses

Statistical analyses were performed by R (http://www.r-project.org/). The clinical parameters used for modelling were age, tumour size, histological subtype, myometrial depth of invasion, LVI, FIGO grade, lymph nodes positive, recurrence, status, recurrence time, and survival time. Univariate and multivariate analysis was performed. To test the association between the biomarker and the clinical parameters, Fisher's exact test was performed for categorical parameters, and a logistic regression model was used for continuous parameters. For survival analysis, overall survival (OS) was censored at the time of last follow-up; events were death due to any cause. Disease-free survival (DFS) was censored at the time of last follow-up if disease progression did not occur; events were death due to any cause, disease relapse, and progression. Recurrence-free survival was censored at the time of last follow-up if the recurrence did not occur; events were death due to any cause and recurrence. The Kaplan–Meier method with log-rank test was used to calculate the cumulative survival time and check the OS, RFS, and DFS difference between the two different Pax8 groups. All reported *P*-values are two-sided. *P*-value was considered significant if $P<0.05$.

RESULTS

The clinical and pathological features of 229 patients with endometrial adenocarcinoma are summarised in Table 1. The patients' ages ranged from 29 to 97 years (median 65). Overall, 72% of cases were type I and 28% were type II subtype. In total, 42% of cases were FIGO G1, 20% G2, and 38% G3. As for tumour stage, 64% of cases were FIGO stage I, 14% stage II, 15% stage III, and 7% stage IV. Tumour size was considered as zero when no gross tumour was identified. In all, 65.5% of patients had lymph

Table 1 Clinical and pathological features of 229 patients with endometrial cancer

	No. of cases	%
No. of evaluable patients	229	
Age (years)		
Median	65	
Range	(29, 97)	
Myometrial invasion (%)		
Median	30	
Range	(0, 100)	
Tumour size (cm)		
Median	4	
Range	(0, 33)	
Tumour subtype		
Type II	64	28
Type I	165	72
Grade FIGO		
1	96	42
2	46	20
3	87	38
Grade nuclear		
1	80	35
2	59	26
3	90	39
Recurrence		
No	177	77
Persistent	10	4
Progression	3	1
Yes	39	17
Pax8 immunoexpression		
Negative	158	69
Positive	71	31
LVI		
No	161	70
Yes	68	30
Tumour stage		
I	146	64
II	31	14
III	35	15
IV	17	7
Lymph node positive		
Not applicable	79	34
No	118	52
Yes	32	14
Disease status		
Alive with no evidence of disease	159	69
Alive with evidence of disease	19	8
Dead of unkown cause	1	0
Dead no evidence of disease	13	6
Dead of disease	32	14
Dead with evidence of disease	4	2
Lost for follow-up	1	0

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nodes dissection. Of these, 21.3% patients had positive lymph nodes and 78.6% had negative lymph nodes.

Univariate analysis of the distribution of clinical factors in relation to dichotomous status of Pax8 expression is illustrated in Table 2. There was a positive association between Pax8$^+$ and high tumour grade ($P=0.002$), presence of LVI ($P<0.019$), and type II tumour subtype ($P<0.0001$). Serous and clear cell carcinomas as well as carcinosarcoma were more likely to express Pax8 than endometrioid adenocarcinomas. Pax8 expression is negatively associated with OS ($P=0.0097$) and DFS ($P=0.019$). However, these significant results were not found in multivariate analysis (data not shown).

Kaplan–Meier survival analysis was conducted. There was an association of Pax8 with OS ($P=0.01486$) with 5-year OS probability of 80.04% for patients with Pax8 − and 55.9% for patients with Pax8$^+$ (Figure 1). There was also an association of Pax8 and DFS probability ($P=0.02028$) with 5-year DFS probability of 72.12% for patients with Pax8 − *vs* 49.88% for patients with Pax8$^+$ expression (Figure 2). Finally an association of Pax8$^+$ and shorter RFS was also seen ($P=0.00203$), with 74.36% for Pax8$^-$ patients and 52.11% for Pax8$^+$ patients (Figure 3).

Table 2 Univariate analysis of the association of Pax8 and the clinicopathological variate

	Pax8 negative	Pax8 positive	*P*-value	Odds ratio
Age	63.04[a]	69.58	0.0003	1.045435
Tumour size	4.77	4.29	0.3674	0.962615
Myometrial invasion	40.95	39.3	0.7384	0.998615
Grade FIGO			0.0024	2.168619
1	74 (46.84)[b]	22 (30.99)		
2	36 (22.78)	10 (14.08)		
3	48 (30.38)	39 (54.93)		
Grade nuclear			0.0006	
1	66 (41.77)	14 (19.72)		
2	42 (26.58)	17 (23.94)		
3	50 (31.65)	40 (56.34)		
Tumour stage			0.3190	
I	105 (66.46)	41 (57.75)		
II	17 (10.76)	14 (19.72)		
III	24 (15.19)	11 (15.49)		
IV	12 (7.59)	5 (7.04)		
Tumour subtype			<0.0001	0.256816
Type II	30 (18.99)	34 (47.89)		
Type I	128 (81.01)	37 (52.11)		
Recurrence			0.0363	2.168619
No	127 (85.81)	50 (73.53)		
Yes	21 (14.19)	18 (26.47)		
LVI			0.0186	2.099539
No	119 (75.32)	42 (59.15)		
Yes	39 (24.68)	29 (40.85)		
Lymph node			1.00	1.070446
No	72 (79.12)	46 (77.97)		
Yes	19 (20.88)	13 (22.03)		
Disease status			0.0096	2.364434
Alive	131 (82.91)	47 (67.14)		
Dead	27 (17.09)	23 (32.86)		
Disease status			0.0189	2.07943
Alive with no evidence of disease	118 (74.68)	41 (58.57)		
Others	40 (25.32)	29 (41.43)		

Continuous variables are tested by logistic regression and categorical variables are tested by the Fisher test. [a]Mean. [b]Count (%).

DISCUSSION

The *Pax8* is located at the 2q13 chromosome band. It is a member of the pair-box family of genes that are required for the formation of several tissues from all germ layers in the mammalian embryo (Poleev *et al*, 1990; Mittag *et al*, 2007). Particularly, *Pax8* gene is

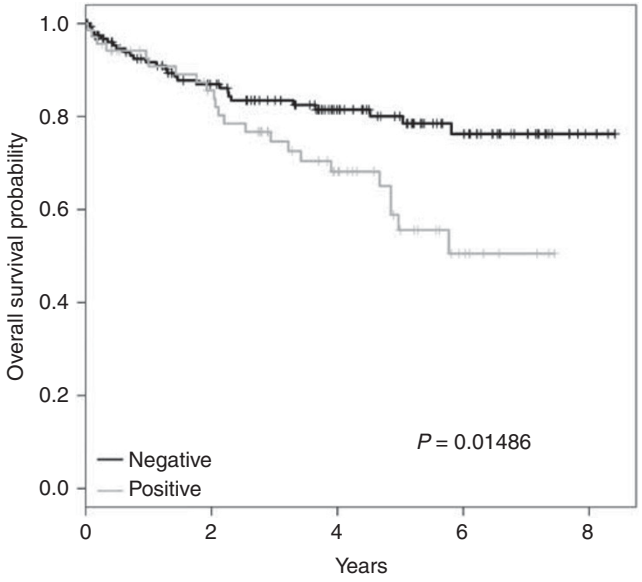

Figure 1 Kaplan–Meier survival analysis revealed the association of Pax8 with overall survival ($P=0.01486$). The black curve is for Pax8$^-$ patients and the grey curve is for Pax8$^+$ patients. The 5-year overall survival probabilities for the Pax8$^-$ and Pax8$^+$ patients were 80.04 and 55.59%, respectively.

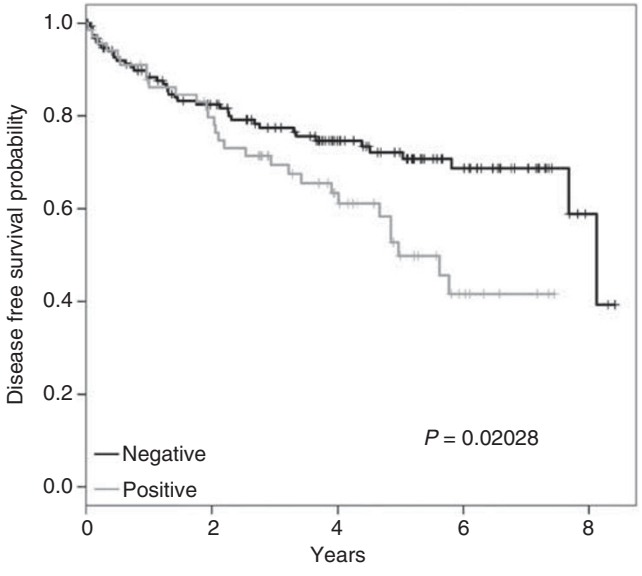

Figure 2 Kaplan–Meier survival analysis revealed the association of Pax8 with DFS probability ($P=0.02028$). The black curve is for Pax8$^-$ patients and the grey curve is for Pax8$^+$ patients. Five-year DFS probability of 72.12% for patients with Pax8$^-$ *vs* 49.88% for patients with Pax8$^+$ expression.

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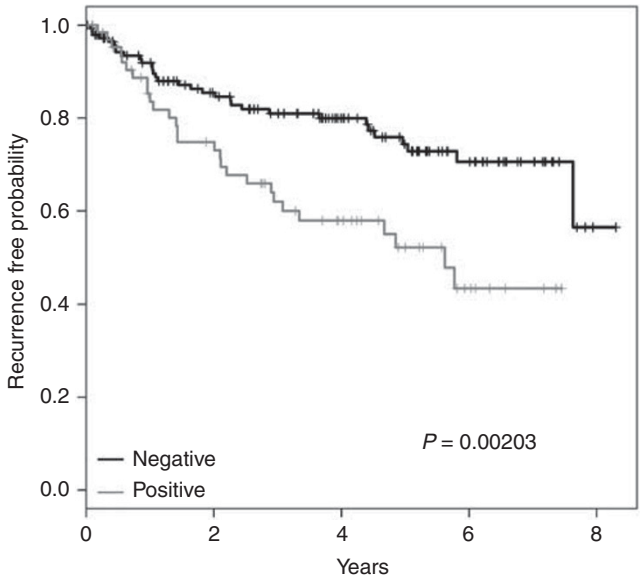

Figure 3 Kaplan–Meier survival analysis revealed the association of Pax8 with recurrence-free probability ($P = 0.00203$). The black curve is for $Pax8^-$ patients and the grey curve is for $Pax8^+$ patients. The 5-year recurrence-free probability for the $Pax8^-$ and $Pax8^+$ patients are 74.36 and 52.11%, respectively.

essential for the organogenesis of the mullerian system, thyroid, and kidney. The biological significance of Pax8 protein expression in cancer is unknown, but recent studies suggest a possible role of Pax8 in tumourigenesis (Castro *et al*, 2006; Cheung *et al*, 2011). In a previous study of human endometrial cancer samples, we found an upregulation of *Pax8* gene in advanced stage USC *vs* early stages USC (fold-change = 1.44) and an upregulation of *Pax8* in USC with bad outcome *vs* USC with good outcomes (fold-change = 1.72), although statistical significance was not reached ($P > 0.05$) possibly due to our small sample size ($n = 5$) (Mhawech-Fauceglia *et al*, 2010, 2011). In this study, we sought to identify the expression and prognostic value of Pax8 protein expression in a large series of human endometrial cancer cases.

On univariate analysis, Pax8 protein-positive expression was associated with clinical and pathological predictive factors of poor outcome, such as high tumour grade, histological subtypes II, and presence of LVI. The association of Pax8 with tumour grade, and histologic type was seen in a previous study (Brunner *et al*, 2011). We also found that patients with tumours expressing Pax8 were more likely to die form disease. The 5-year OS probability for patients with $Pax8^-$ was better than those with $Pax8^+$ (80.04 *vs* 55.9%). Also, patients with $Pax8^-$ have better 5-year DFS (72.12% for patients *vs* 49.88%) and better 5-year RFS (74.36% *vs* 52.11%) in comparison with those patients with $Pax8^+$ tumours. These findings validate results of our prior study and provide evidence that overexpression of Pax8 protein could be a potential biomarker for poor outcome in patients with endometrial cancer.

Recently, Hahn and group showed an overexpression of Pax8 in numerous cancer cell lines, including ovarian and endometrial cancer (Cheung *et al*, 2011). In addition, when untested shRNAs against Pax8 were introduced to ovarian, kidney, and endometrial cancer cell lines, a drop in cell viability in cells that overexpressed Pax8 was noted, suggesting that inhibition of Pax8 activity could have a substantial impact on cancer treatment. Owing to high expression of Pax8 by type II tumours, which can be resistant to traditional chemotherapy, tailored targeted therapy to silence Pax8 could be a promising therapeutic option for these cases. Mechanistic studies are being conducted in our laboratory to define the role of Pax8 in cell culture.

In summary, our results indicate that endometrial cancer patients with tumours expressing $Pax8^+$ have worse clinical outcomes compared with those with $Pax8^-$. $Pax8^+$ expression pattern is found to be negatively associated with 5-year OS, 5-year DFS, and 5-year RFS probabilities. These findings support the potential clinical utility of incorporating Pax8 IHC in the evaluation of endometrial cancer patients in order to identify those who are at higher risk of early recurrence. Silencing Pax8 could be the target of a new generation of therapeutic agents for selected patients.

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Conflict of interest

The authors declare no conflict of interest.

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