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# Phosphorylated KDR expression in endometrial cancer cells relates to HIF1α/VEGF pathway and unfavourable prognosis

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**Vascular endothelial growth factor (VEGF) is a potent angiogenic factor for many malignant neoplasms exerting its function through activation of specific membrane receptors, that is, KDR/flk-1, residing in endothelial cells. Several recent reports indicate that VEGF receptors are also expressed in cancer cells, suggesting that specific VEGF-originated cancer cell reactions may parallel the endothelial response. Using a novel monoclonal antibody, recognizing the activated (phosphorylated) form of the KDR receptor (pKDR), we assessed the expression of pKDR in normal and malignant endometrium. A strong and consistent cytoplasmic and nuclear pKDR expression was noted in the normally cycling endometrium, including epithelial, stromal and endothelial cells, suggesting a role in the normal menstrual cycle. Approximately, one-third of the 70 stage I endometrioid adenocarcinomas analysed exhibited an intense cytoplasmic and nuclear pKDR expression in both cancer cells and peritumoral vessels. It was noted that such pKDR reactivity in cancer cells was related directly to VEGF, VEGF/KDR complexes and HIF1α (hypoxia inducible factor 1α) expression. Furthermore, pKDR expression was significantly associated with poor prognosis. It is concluded that the VEGF/KDR pathway is activated in both normally cycling and malignant endometrium, suggestive of an important role in the biology of this tissue. The unfavourable prognosis that VEGF confers to endometrial adenocarcinomas could be attributed to its angiogenic activity, but also to a direct effect on cancer cells through an autocrine VEGF/KDR loop.**
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Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor that promotes endothelial cell proliferation and affects survival.[1,2] It acts on specific tyrosine kinase receptors, VEGFR-1 (*flt-1*), VEGFR-2 (KDR/*flk-1*) and VEGFR-3 (*flt-4*). Although these molecules are predominantly expressed on endothelial cells, the VEGFR-1 receptor has also been identified in trophoblastic cells, renal mesangial cells and monocytes, while VEGFR-2 expression has been detected in haematopoietic stem cells and megakaryocytes.[3–6] Both receptors are glycosylated and, in this form, undergo phosphorylation, in response to VEGF, which is an important step in the signalling of VEGF.[7,8]

Cancer cells and intratumoral endothelium have also been reported to express VEGF receptors.[9–13] The expression of VEGFRs, however, does not necessarily mean active participation in cancer or endothelial cell biology as this can only be analysed by assessing the activated forms of these receptors. Brekken *et al*[14] produced monoclonal antibodies that preferentially recognize VEGF bound to its receptor KDR, which presumably reflects the activated KDR receptor. Indeed, expression of VEGF/KDR complexes were noted in cancer cells and intratumoral vasculature in lung and endometrial carcinomas and this feature was significantly related to prognosis and increased vascular density at the invading tumour front.[15,16]

Recently, specific monoclonal antibodies have been raised against the phosphorylated (activated)

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form of KDR (pKDR) by our group.[17,18] In the current study, we used one of these antibodies to investigate the expression of the pKDR receptor in normal and malignant endometrium. Association with histopathological features, angiogenesis, hypoxia-regulated proteins and patients' prognosis was also assessed.

## Materials and methods

We examined 70 tumour samples from patients with stage I endometrioid adenocarcinomas, and 20 samples from normally cycling endometrium of both proliferative and secretory phase. Formalin-fixed, paraffin-embedded material was retrieved from the archives of the Department of Pathology, Democritus University of Thrace Medical School, Alexandroupolis, Greece. All patients had been treated surgically with total abdominal hysterectomy and bilateral salpingo-oophorectomy. No lymph node sampling of the iliac nodes was performed, and N-staging was based on pelvic and abdominal CT scan. Histological typing and grading of the endometrial tumours (grade 1 *vs* grades 2 and 3) and the depth of myometrial invasion ($<1/2$ *vs* $>1/2$) were assessed on haematoxylin–eosin sections, using standard criteria.[19,20] Lymphatic-vascular space invasion was recorded as being present if tumour cells were seen within a space with a definite and clearly identifiable endothelial lining. The follow-up of patients ranged from 6 to 170 months with a mean of 66 months. For patients alive at the time of analysis (61/70 patients), the median follow-up was 70 months (range 22–176).

### Immunohistochemistry for pKDR

The pKDR protein was assessed using monoclonal antibody 34a raised against the Y1214 tyrosine residue of the KDR protein.[17]

Sections were deparaffinised and peroxidase was quenched with methanol and $H_2O_2$ 3% for 15 min. Thereafter, slides were placed in antigen unmasking buffer, pH 6.0 (code: TAR001, ILEM, Italy) and microwaving followed ($3 \times 4$ min). The primary antibody (supernatant dilution 1:2) was applied overnight, at room temperature. Following washing with TBS, sections were incubated with a secondary mouse anti-rabbit antibody (Kwik Biotinylated Secondary, 0.69A Shandon-Upshaw, Pittsburgh, PA, USA) for 15 min and washed in TBS. Kwik Streptavidin peroxidase reagent (039A Shandon-Upshaw, Pittsburgh, PA, USA) was applied for 15 min and sections were again washed in TBS. The colour developed by 15 min of incubation with DAB solution and sections were weakly counterstained with haematoxylin. Normal kidney sections were used as positive controls.[18] Normal immunoglobulin-G was substituted for the primary antibody as the negative control, at a concentration where immunostaining of control slides gave a faint cytoplasmic staining.

The percentage of cancer cells with cytoplasmic and nuclear pKDR reactivity was recorded separately after inspection of all fields in the tissue sample. The percentage of positive cells was recorded in each individual field and the median value obtained for each case was the final score.

### Other Immunohistochemistry

Table 1 shows the antibodies and details of the immunohistochemical procedures used to detect the expression of various oncoproteins and growth factors/receptors. Extensive reports of the methods used have been published previously.[16,21–23]

The assessment of HIF1α and 2α expression was performed according to an HIF grading system reported previously.[21] Scoring the expression of VEGF/KDR complexes, VEGF and TP were based on assessing the percentage of cancer cells with cytoplasmic VEGF/KDR or VEGF expression and nuclear TP expression, following examination of the whole tumour area at $\times 200$ magnification.[16,22] The median value was used to score cases with low or high reactivity. A 10% cancer cell positivity was required to score a case as positive for bcl-2 protein cytoplasmic expression, p53 protein nuclear accumulation and nuclear oestrogen (ER) and progester-

**Table 1** Details of the antibodies, dilutions and antigen retrieval methods used in this study

| *Primary antibody* | *Dilution/incubation time* | *Antigen retrieval* | *Specificity* | *Source* | *Reference* |
|---|---|---|---|---|---|
| JC70 (CD31) | 1:50 (60 min[a]) | Protease XXIV | Endothelium | Dako, Denmark | Giatromanlaki *et al*[16] |
| ESEE 122 | 1:20 (90 min[a]) | MW | HIF-1α | Oxford University | Sivridis *et al*[19] |
| EP 190b | Neat (90 min[a]) | MW | HIF-2α | Oxford University | Sivridis *et al*[19] |
| VG1 | 1:4 (90 min[a]) | MW | VEGF | Oxford University | Sivridis and Giatromanlaki[20] |
| 11B5 | 1:3 (60 min[a]) | MW | VEGF/KDR | Texas University | Giatromanlaki[16] |
| P-GF.44C | 1:4 (60 min[a]) | No | TP | Oxford University | Sivridis and Giatromanlaki[20] |
| DO-7 | 1:30 overnight at 4°C | MW | p53 | Dako | Sivridis *et al*[21] |
| 124 | 1:80 overnight at 4°C | MW | bcl-2 | Dako | Sivridis *et al*[21] |
| 1D5 | 1:100 (60 min[a]) | MW | ER | Immunon-Shandon | Sivridis *et al*[19] |
| 1A6 | 1:100 (60 min[a]) | MW | PgR | Immunon-Shandon | Sivridis *et al*[19] |

MW = microwave heating.
[a]At room temperature.

one (PgR) receptor reactivity, which are the generally accepted cutoff points for these antibodies.[21,23]

Microvessel counting was used for angiogenesis assessment. Sections were scanned at low power and afterwards at $\times 200$ fields in order to group cases into three categories (low, medium and high). Areas at the invading tumour edge of the highest vascularization were chosen at low power ($\times 100$) and microvessel counting followed on three chosen $\times 200$ fields of the highest density. The vascular density (VD) was the mean of the vessel counts obtained in these three fields. Vessels with a clearly defined lumen or well-defined linear vessel shape but not single endothelial cells were taken into account for microvessel counting. The median value was used to define two groups of low and high VD.[16]

### Statistical Analysis

Statistical analysis and graphic presentation were performed using the GraphPad Prism® 4.0 package and the Instat® 3.0 packages (GraphPad, San Diego CA, www.graphpad.com). The Fisher's exact test, the chi-square *t*-test or the unpaired two-tailed *t*-test was used for testing relationships between categorical variables as appropriate. Spearman analysis was used to assess correlation between continues variables. Survival curves were plotted using the method of Kaplan–Meier, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumour variables on overall survival. A *P*-value $<$ or equal to 0.05 was considered significant.

## Results

### pKDR in the Normal Endometrium

pKDR was expressed strongly in the normally cycling endometrium. The staining was both cytoplasmic and nuclear in the glandular epithelial cells and extended throughout the menstrual cycle (Figure 1a). The stromal cells showed only nuclear reactivity; this was limited only to the functional layer during the proliferative phase but involved uniformly the basal and functional layers in the secretory phase endometrium. The myometrial cells showed strong cytoplasmic positivity. The endothelium showed cytoplasmic and nuclear pKDR expression in all uterine coats: endometrium, myometrium and perimetrium (Figure 1b). Lymphatic vessels of the myometrium were also strongly reactive.

### pKDR in Endometrial Cancer

Strong cytoplasmic reactivity was noted in 27/70 endometrial carcinoma cases (Figure 1c); the reaction ranged from 20 to 80% (median 55%) in cancer cells. In 8/27 cases with cytoplasmic positivity, nuclear pKDR reactivity was also noted in $>10\%$ of the neoplastic nuclei (Figure 1d).

Expression of pKDR was also noted in rather larger peritumoral vessels, mainly at the invading tumour front (Figure 1e), while very small immature blood capillaries were negative.

### Correlation with Histopathological Variables

Table 2 shows the relation of pKDR expression with histological variables and hormone receptor status. There was no association with histological grade and depth of myometrial invasion, while a marginal association with nuclear expression of progesterone receptors was noted.

### Correlation with Molecular Variables and VD

Table 3 shows the association of pKDR expression with angiogenesis, hypoxia-inducible factors and oncoprotein expression. pKDR was directly linked with HIF1α and VEGF expression ($P=0.01$ and 0.001, respectively). A strong association of pKDR expression with the expression of the VEGF/KDR complex in the cytoplasm of cancer cells was noted. No association with HIF2α, vascular density, TP, p53 and bcl-2 protein expression was noted.

### Survival Analysis

Overall, nine patients died during the follow-up of patients, corresponding to a 12.8% death rate. The death rates were 2/43 (4.6%) and 7/27 (25.9%) in cases with high and low pKDR expression, respectively ($P=0.02$). Out of the traditional histology prognostic features, histology grade was the most important in death rate analysis (the death rates were 4/55 (7.2%) for grade 1 *vs* 3/15 (20%) for grade 2/3 cases; $P=0.16$).

Survival analysis according to Kaplan–Meier showed a significant association of pKDR expression in cancer cells with poor overall survival ($P=0.009$) in stage I endometrial cancer patients (Figure 2a). Figure 2b shows that the significant worse prognosis of patients with high pKDR expression is also maintained in the group of patients with grade 1 histology. In multivariate analysis (Table 4) taking into account the pKDR and the histological variables, pKDR was a strong and independent marker of prognosis ($P=0.007$, risk ratio 2.75). Taking all parameters into account, HIF1α and histology grade were also independently linked with prognosis.

## Discussion

VEGF is a potent angiogenic growth factor promoting endothelial cell proliferation and new blood vessel formation;[1,2] its expression in cancer cells has been associated with increased vascular density and unfavourable survival in a variety of malignant

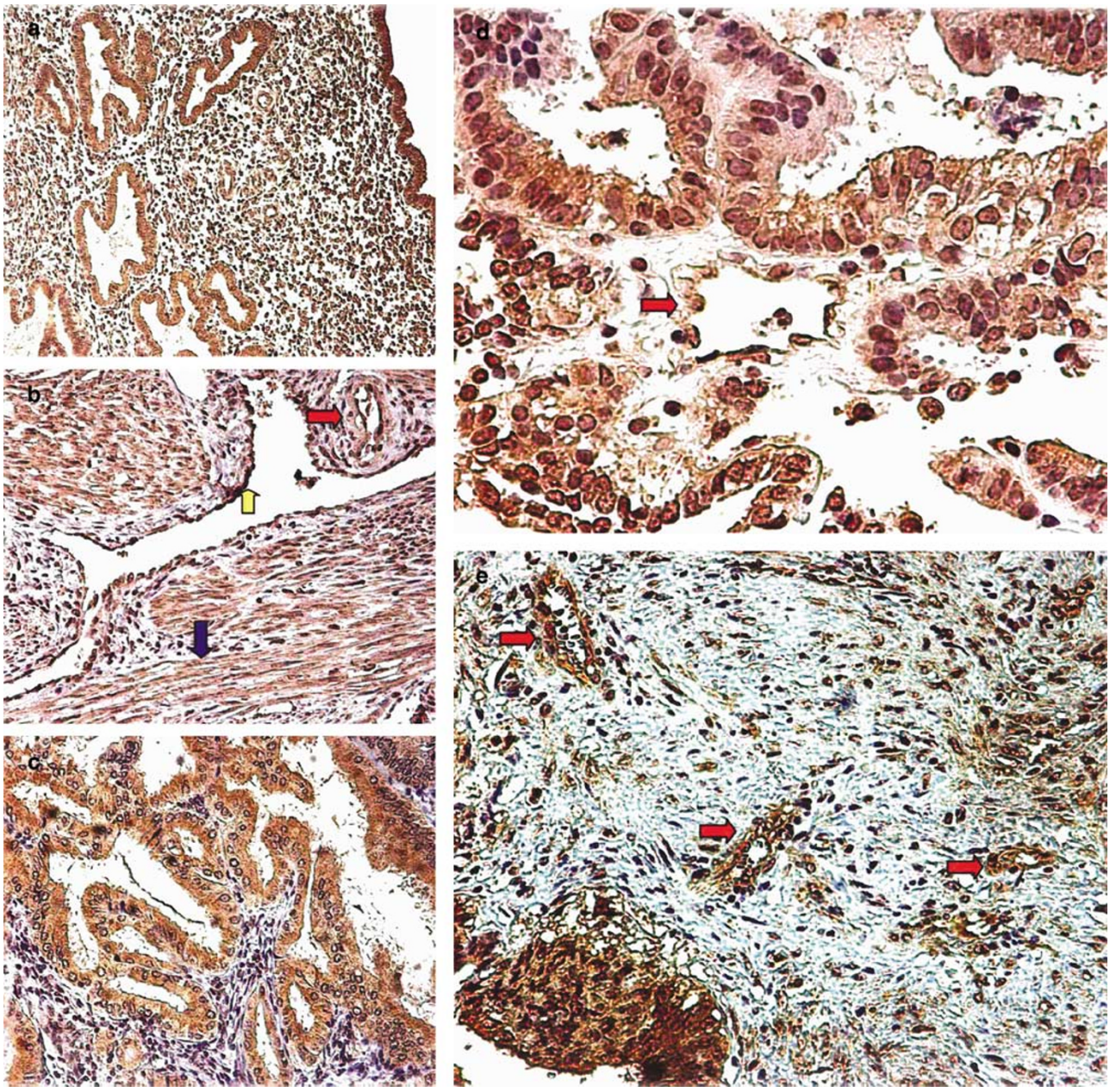


**Figure 1** pKDR expression in normal and neoplasic endometrium: (**a**) pKDR cytoplasmic and nuclear expression in normal proliferating endometrium and stroma cells. (**b**) pKDR expression in normal myometrium (blue arrow) and related lympatics (yellow arrow) and vessels (red arrow). (**c**) Cytoplasmc pKDR expression in endometrioid cancer. (**d**) pKDR nucelar expression in endometrioid cancer and intratumoral vessel (red arrow). (**e**) pKDR expression in tumour-related vasculature in the invading tumour edge (red arrows).

tumours. Such a relation between VEGF expression, angiogenesis and prognosis was reported by us previously in patients with endometrial cancer.[22] Using a monoclonal antibody, recognizing the VEGF/KDR complexes, we also verified overexpression in cancer cells and in capillary endothelium located at the invading tumour edge.[16] Cancer cell reactivity was thought to represent VEGF content, as KDR was thought to be confined to endothelial cells, despite reports suggesting the presence of KDR in a variety of human tumours, including breast, lung and endometrial carcinomas.[9–13] Constitutive expression of KDR, however, may not necessarily mean an active KDR pathway.

It was only recently that monoclonal antibodies were raised against the phosphorylated form of KDR allowing reconsideration of the role of KDR in tissues.[17,18] The wide distribution of pKDR in normal and malignant epithelial cells and its expression in both cytoplasm and nuclei stressed the importance of the VEGF/KDR pathway in epithelial cell biology.

In this study, we examined the expression of the phosphorylated (activated) KDR receptor in normal

**Table 2** Association of pKDR expression with histological parameters and hormone receptor expression in endometrial adenocarcinomas

| | *pKDR* | | P-*value* |
|---|---|---|---|
| | *Low* | *High* | |
| *Histological grade* | | | |
| 1 | 33 | 22 | 0.76 |
| 2,3 | 10 | 5 | |
| *Myometrial depth invasion* | | | |
| <1/2 | 23 | 13 | 0.80 |
| >1/2 | 20 | 14 | |
| *ER* | | | |
| Low | 35 | 20 | 0.77 |
| High | 9 | 7 | |
| *PgR* | | | |
| Low | 35 | 21 | 0.08 |
| High | 8 | 6 | |

ER, oestrogen receptor; PgR, progesterone receptor; pKDR, phosphorylated form of the KDR.

**Table 3** Association of pKDR expression with molecular variables expressed in endometrial cancer cells

| | *pKDR* | | P-*value* |
|---|---|---|---|
| | *Low* | *High* | |
| *HIF1α* | | | |
| Low | 28 | 9 | 0.01 |
| High | 15 | 18 | |
| *HIF2α* | | | |
| Low | 37 | 20 | 0.22 |
| High | 6 | 7 | |
| *VEGF* | | | |
| Low | 30 | 8 | 0.001 |
| High | 13 | 19 | |
| *VEGF/KDR* | | | |
| Low | 33 | 5 | <0.0001 |
| High | 10 | 22 | |
| *VD invading front* | | | |
| Low | 23 | 12 | 0.62 |
| High | 20 | 15 | |
| *TP nuclear* | | | |
| Low | 34 | 21 | 0.99 |
| High | 9 | 6 | |
| *Mut-p53* | | | |
| Low | 41 | 25 | 0.63 |
| High | 2 | 2 | |
| *Bcl-2* | | | |
| Low | 29 | 19 | 0.99 |
| High | 14 | 8 | |

ER, oestrogen receptor; PgR, progesterone receptor; pKDR, phosphorylated form of the KDR; VEGF, vascular endothelial growth factor; VD, vascular density; HIF2α, hypoxia-inducible factor 1α.

and malignant endometrial tissues. pKDR was consistently expressed in the normal endometrium throughout the menstrual cycle. The subcellular

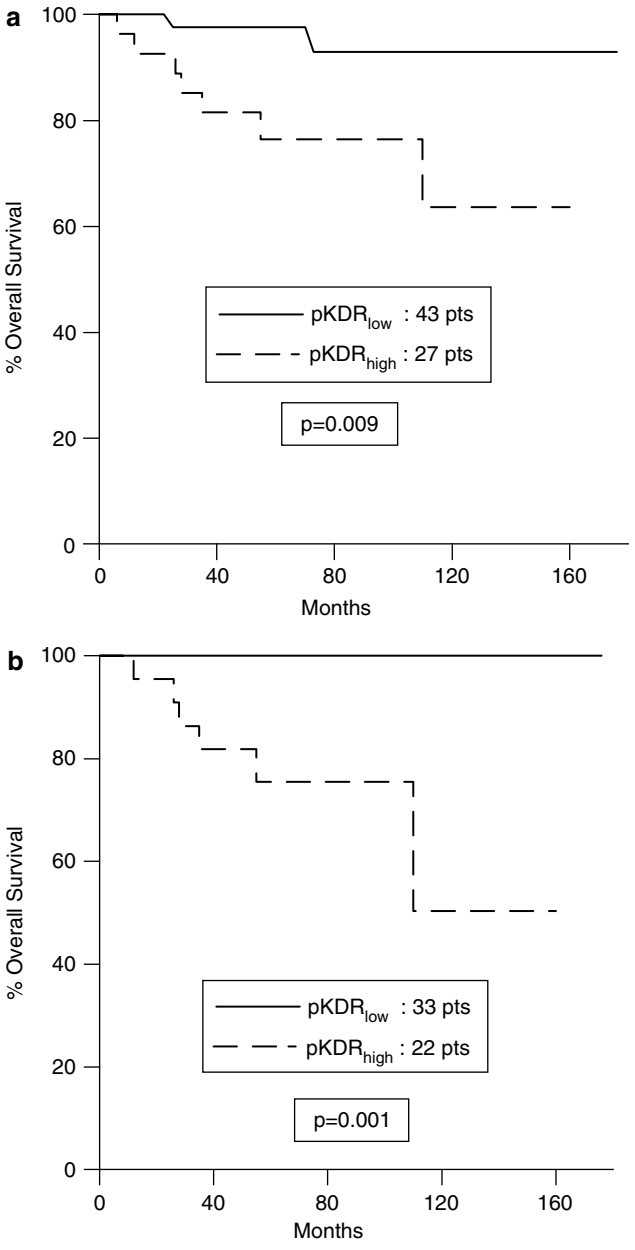


**Figure 2** Kaplan–Meier survival curves stratified for pKDR expression in endometrial cancer cells (**a**, all cases; **b**, cases with grade histology).

localization indicated a cytoplasmic and nuclear shift of the activated receptor. The cyclic expression of pKDR in the stromal cells of the basal layer of the endometrium (switched on in the secretory and off in the proliferative phase) shows that the VEGF/KDR pathway may have a role in the cyclic regeneration and degeneration of the normal endometrium. VEGF is expressed in normal endometrial stromal cells, an expression that appears to be increased in the presence of ovarian steroids,[24,25] specifically progesterone, as suggested in the current study.

Expression of pKDR was also observed in the cytoplasm and nuclei of endometrial cancer cells in

**Table 4** Multivariate analysis of death events taking into account all histological and molecular variables studied (Model 1) or pKDR and morphological variables alone (Model 2)

| *Variable* | *Model 1* | | *Model 2* | |
|---|---|---|---|---|
| | t-*ratio* | P-*value* | t-*ratio* | P-*value* |
| pKDR | 1.12 | 0.26 | **2.75** | **0.007** |
| Grade | **2.14** | **0.03** | 1.16 | 0.24 |
| Depth | 1.64 | 0.10 | 0.75 | 0.45 |
| Vasc. invas. | 1.46 | 0.14 | 0.54 | 0.59 |
| HIF1α | **2.09** | **0.04** | — | — |
| HIF2α | 0.65 | 0.51 | — | — |
| VEGF | 1.54 | 0.12 | — | — |
| VD | 1.70 | 0.09 | — | — |
| p53 | 0.71 | 0.47 | — | — |
| bcl-2 | 0.34 | 0.72 | — | — |
| PgR | 0.24 | 0.80 | — | — |
| ER | 1.59 | 0.11 | — | — |

ER, oestrogen receptor; PgR, progesterone receptor; pKDR, phosphorylated form of the KDR; VEGF, vascular endothelial growth factor; VD, vascular density; HIF2α, hypoxia-inducible factor 1α.
The statistically significant values are in bold.

approximately 30% of the cases analysed. This paralleled the expression of the VEGF/KDR complexes in the cancer cell cytoplasm, indicating that KDR is not simply expressed in endometrial cancer cells but also participates actively in the biology of this neoplasm. A direct association between the expression of pKDR and VEGF is noted, suggestive of an autocrine loop, where cancer cells by secreting VEGF activate a KDR signalling pathway. The existence of such an autocrine mechanism has been also suggested previously in a number of studies.[26–29] Whether such an autocrine loop functions as a stimulus for cancer cell proliferation, resistance to apoptotic stimuli or through some other process remains obscure.[30–33] Nevertheless, expression of pKDR was associated significantly with poor prognosis of stage I patients. Histological grade was also a significant independent variable of prognosis, while depth of myometrial invasion and lympho-vascular space invasion did not reach significance. The finding that pKDR expression defined a group of poor prognosis even in the grade 1 cases supports the suggestion that the VEGF/KDR route contributes to the development of a particularly aggressive endometrial tumour phenotype. This is probably achieved by two main pathways: (a) manipulation of cancer cell behaviour through autocrine cancer cell routes and, (b) accentuation of the angiogenic process (as previously shown.[16,22])

The concurrent expression of pKDR and HIF1α in many endometrial adenocarcinomas could be a direct result of VEGF upregulation by HIF1α and subsequent activation of KDR. Yet, a functional association of HIF1α with KDR upregulation cannot be entirely excluded. Gerber *et al*[34] showed that, in contrast to the *flt*-1 receptor, KDR is not induced by hypoxia. However, this finding contrasts to Waltenberger *et al*'s[35] earlier report, where hypoxia upregulated the expression of KDR in the endothelial cells of umbilical veins. Upregulation of VEGF and KDR, which is paralleled by HIF1α expression, has also been recorded in rabbit skeletal muscle during acute hypoxia.[36] KDR is also induced by hypoxia in choroid-retinal endothelial (RF/6A) cells.[37] In any case, the co-expression of pKDR with the HIF1α downstream genes may be an additional reason for the unfavourable prognosis noted in tumours with pKDR expression. The nuclear role of pKDR need further evaluation, but a nuclear role for other tyrosine kinase receptors (ie erbB4) has recently been shown.[38]

It is concluded that the VEGF/KDR pathway, in contrast to previous beliefs, is activated in the normally cycling endometrium and in more than 30% of endometrial adenocarcinomas, suggesting an important role in the biology of normal endometrium and endometrial neoplasia. The unfavourable prognosis of VEGF-expressing endometrial carcinomas could be attributed both to the angiogenic activity of the factor and its direct effect on cancer cells, probably through an autocrine VEGF/KDR mechanism. Specific therapies targeting KDR phosphorylation (anti-VEGF monoclonal antibodies or tyrosin kinase inhibitors) may therefore posses a dual activity both by suppressing angiogenesis and by interfering with the biology of cancer cells.

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