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ORIGINAL ARTICLE

Expression of Delta Like Ligand 4 (DLL4) in endometrial carcinomas and tumor vasculature

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Summary

Purpose: *Delta like ligand 4 (DLL4) is a transmembrane ligand of the Notch Signalling pathway, that regulates blood vessel sprouting and maturation. We investigated the expression of DLL4 in endometrial cancer.*

Methods: *DLL4 was assessed in the plasma (with ELISA) and tissues (with immunohistochemistry) 33 patients with endometrial cancer, treated with radical hysterectomy for stage I endometroid carcinoma. The angiogenic activity (AA) of endometrial cancer was quantified by assessing the CD31+ microvessel density (MVD) in the invading tumor front. Vascular maturation index (VMI), defined as the percentage of CD31+ microvessels expressing DLL4, was calculated as the ratio of the CD31+ MVD to the DLL4+ MVD.*

Results: *The angiogenic activity was directly related with the histological grade (p=0.01). The VMI ranged from 0.1 to 0.7 (median 0.34). The concentration of DLL4 in the plasma ranged from 55-81pg/ml (mean 62.8) before, and dropped to 55-62 (mean 58.2) after hysterectomy (p<0.05). DLL4 was also expressed by cancer cells in 17/33 cases. No correlation between DLL4-related parameters with histopathological variables was noted.*

Conclusion: *This pilot study shows that DLL4 is overexpressed in endometrial cancer cells, vasculature and is also elevated in the plasma of a fraction of patients before surgery. The percentage of DLL4+ vessels in the penetrating sample ranged from 10-70%, indicating a large difference in the quality of angiogenesis produced between the endometrial tumors of the same histological type and differentiation.*

Key words: *endometrial cancer, angiogenesis, CD31, DLL4*

Introduction

Cancer angiogenic ability is essential for the tumor spread to neighboring and distant organs. The progression of the vascular network is responsible for the metastatic development of cancer tissues. Tumor cells can perforate both the lymphatic and blood vessels growing at divergent tissues, circulating the intravascular stream, and developing the relevant metastasis risks [1]. Both angiogenesis representing new blood vessels and lymphangiogenesis, referring to the formation of the lymphatic vessel, contribute to the development of tumor as well as its progression. Both of them have a responsibility in the formation of the vascular network responsible for removing waste products and supplying immune cells, nutrients and oxygen [2]. The newly formed vasculature, mainly a result of VEGF overexpression, results in an immature irregular network with high vascular permeability, easily collapsing walls and a chaotic architecture that compromises the blood flow [3,4]. Lack of vascular

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maturation may result in poor tumor perfusion and hypoxia, even in highly angiogenic tumors.

The Notch signaling pathway is an important regulator of the maturation of the tumor vasculature [5]. In this pathway, 4 transmembrane receptors (Notch 1,2,3 and 4) on the endothelium are activated by five ligands (Jagged 1 and 2 and Delta-like ligand DLL1,3 and 4). DLL4 ligand prevents the formation of the 'tip cell phenotype' characterizing the sprouting endothelial cells, and promotes stalk cell formation and vascular maturation [6]. DLL4 provides a negative feedback effect on VEGFR2 expression, blocking the formation of immature vessels driven by the VEGF/VEGFR2 axis.

In the current study we assessed the expression of notch ligand DLL4 in endometrial cancer and related vasculature. The plasma levels of DLL4 was also assessed in patients.

Methods

Thirty-three patients with endometrial cancer, treated with radical hysterectomy for stage I endometroid carcinoma, were prospectively recruited in the study.

Study approval

The Scientific and Ethics Research Committees of the University Hospital of Alexnadroupolis granted approval for the conduct of this study (ΕΣ8/01-12-2016). All personal data of the participants were fully secured during all the procedures of participants selection, data collection and analysis, while all applicable provisions for personal data in accordance with the applicable provisions in Greece and EU Regulation 2016/679 were followed. All patients gave written informed consent form.

Patients and disease characteristics

Paraffin embedded tissue material from 33 endometrial carcinomas of endometrioid histology was retrieved from the archives of the Department of Pathology, Democritus University of Thrace. All patients had undergone radical hysterectomy and were of FIGO stage I. Regarding tumor differentiation, 19/33 were of grade 1, while 9/33 and 5/33 were of grade 2 and 3, respectively. In 7/33 cases there was a deep myometrial invasion exceeding the 50% of the myometrium thickness. Extension of the tumor to the endocervix was noted in 6/30 cases. The age of patients ranged from 33 to 82 years (median 64).

Immunohistochemistry

For DLL4 immunohistochemical detection we used the NB600-892 rabbit polyclonal DLL4 antibody (Novus Biologicals, Cambridge, UK). For the assessment of the panendothelial cell marker CD31 we used the JC70 monoclonal antibody (Dako, Denmark). The study was performed on 3 μm tissue sections. Sections used for the detection of DLL4 and CD31 antigens were sequential to allow a direct comparison of the same tissue area.

The immunohistochemistry method for the CD31 panendothelial cell marker has been previously reported. For DLL4, the following immunohistochemical procedure was applied. For heat-induced epitope retrieval, the sections were placed in citrate buffer (1:10 dilution, pH 9.0) and heated at 120°C for 3x5 min. The Envision Flex TM, Mouse high pH kit (DAKO, code K8002) was used for immunohistochemistry. Slides were washed with the Envision Flex wash buffer, 3x5 min and they were then incubated overnight at 4°C with the anti-DLL4 primary antibody, diluted at 1:50. After washing, endogenous peroxidase activity was neutralized using the Envision Flex Peroxidase Blocking reagent for 10 min. The slides were washed with the wash buffer for 5 min and then incubated with the secondary antibody (Envision Flex LINKER) for 15 min and were washed again with the Envision Flex wash buffer. Then, the slides were incubated with the Envision Flex/HRP for 30 min and washed again. The colour reaction was developed with the Envision Flex DAB+ Chromogen for 10 min. The sections were counterstained with hematoxylin, dehydrated and mounted. Normal species immunoglobulin-G was substituted for the primary antibody as the negative control. Sections from clear cell renal carcinomas were used as positive controls for DLL4 vessels staining and lack of expression in the cancer cells.

Assessment of the angiogenic activity (AA)

The angiogenic activity (AA) of endometrial cancer was assessed in the invading tumor front, where cancer cells invade the myometrium, in CD31 immunostained tissue sections. Assessment was performed in three optical fields of the highest vascular density. The mean value of these numbers was used to characterize the microvessel density (MVD) in the invading tumor front.

Assessment of the DLL4 vascular maturation index (VMI)

The assessment of the expression of DLL4 by tumor vessels was performed in the same tissue areas (x20 optical fields) chosen for MVD assessment, at the invading tumor front and using the same methodology as applied on anti-CD31 stained tissue sections (parallel tissue sections to allow direct comparison).

The percentage of microvessels expressing DLL4 was calculated as the ratio of the CD31+ MVD to the DLL4+ MVD. This was the DLL4 vascular maturation index (VMI).

Assessment of DLL4 expression in cancer cells

As DLL4 immunostaining showed also cancer cell reactivity, the intensity and extent of staining was scored by counting the percent of cancer cells with strong DLL4 cytoplasmic reactivity in all available x200 optical fields. The mean value was used to score each case.

Dll4 detection in plasma

Blood samples were collected in tubes using heparin as anticoagulant and placed immediately after sampling on a special Histopaque solution (Histopaque®-1077;

SIGMA) and centrifuged at 1x1000g for 30 min at room temperature. The supernatant was collected and the samples were stored at -20°C until analysis. An already certified kit, the ABIN423855 ELISA KIT (antibodies-online.com, CN) was used to detect DLL4 in plasma. The detection range of the DLL4 protein reported in the kit is 0.156 - 10 ng / ml. The lower limit of detection of the assay is 0.054 ng / ml. One hundred microliters of each sample was evaluated in duplicate in a 96-well plate (96 well plate- included in the kit). After 2 h of incubation at 37°C, 100 microliters of detection reagent A was added to each well. This was followed by incubation for 1 h at 370°C. Wash with appropriate buffer, and add 100 of prepared B-detection reagent. After 30 min of incubation at 370°C, 50 ml of stop solution was added. Ninety microliters of substrate solution was added again, and after 20 min incubation at 370°C, 50 ml of stop solution was added. Optical absorption was estimated at 450 nm in FluoStar (Omega-Radiotherapy Oncology Clinic). The 96-well plates were sealed with a cellulose acetate film to prevent evaporation throughout the process. The expression of protein was quantified according to the standard curve and the equation resulting from the absorptions of known protein concentrations (standard curve).

Table 1. Association between the angiogenic activity in the invading tumor front with histopathological variables

	MVD		
	Low	*High*	*p*
Grade			
1	11	8	0.01*
2	5	4	
3	0	5	
Depth, %		0.60	
<50	12	14	
>50	4	3	
Endocervix		0.93	
No	13	14	
Yes	3	3	

*Grade 1,2 vs 3

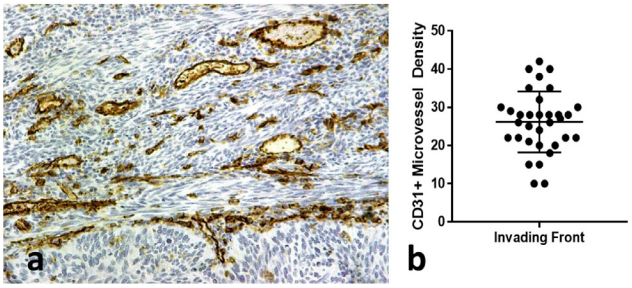

Figure 1. a. Typical immunohistochemical image of high angiogenic activity (CD31+ vessels) in the invading front of an endometrioid carcinoma. **b.** Distribution of the CD31+ MVD in the invading front among endometrial cancer cases.

Statistics

Statistical analysis was performed using the GraphPad Prism 7.0 (GraphPad Software Inc., USA). The unpaired two-tailed t-test or the Wilcoxon matched pairs signed rank test was used to compare groups with continuous variables, as appropriate. Linear regression analysis was applied to assess association between continuous variables. P values<0.05 were considered significant.

Results

Angiogenic activity

The CD31 positive MVD in the invading front ranged from 10-42 microvessels per optical field (median 27) (Figure 1a, 1b).

Table 1 shows the association between the angiogenic activity in the invading tumor front with histopathological variables. Tumors with grade 3 histology were significantly related to high angiogenic activity (p=0.01).

Expression of DLL4 by cancer cells and tumor vessels

DLL4 was expressed by cancer cells in 17/33 cases. The overall expression in terms of the percent of cancer cells positive for DLL4 ranged from 0-100% (median 20%) (Figure 2a). Linear regression analysis revealed no correlation with CD31+ MVD. DLL4 was expressed by tumor vessels (Figure 2b). The DLL4+ MVD ranged from 3-18 (median 9) (Figure 2c). There was no association of DLL4 expression by cancer cells or of the DLL4+ MVD with histopathological variables (Table 2).

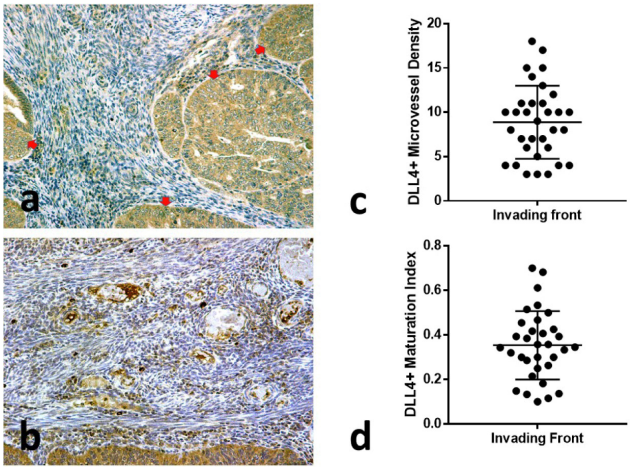

Figure 2. a. High immunohistochemical expression of DLL4 by cancer cells of an endometrial carcinoma. **b.** Immunohistochemical image of DLL4 expressing tumor vessels in the invading front of an endometrioid carcinoma. **c.** DLL4+ mcirovessel density in the invading tumor front of endometrioid carcinomas. **d.** DLL4 maturation index in the invading front of endometrioid carcinomas.

Table 2. Association of DLL4 expression by cancer cells and DLL4+ MVD with histopathological variables

	DLL4+ cancer cells			*DLL4+MVD*		
	Low	*High*	*p*	*Low*	*High*	*p*
Grade						
1	10	9	0.61	11	8	0.12
2	6	3		5	4	
3	2	3		1	4	
Depth, %						
<50	15	11	0.48	14	12	0.60
>50	3	4		3	4	
Endocervix						
No	16	11	0.24	14	13	0.93
Yes	2	4		3	3	

The DLL4 maturation index

The percentage of microvessels expressing DLL4 was calculated as the ratio of the CD31+MVD to the DLL4+MVD in the invading tumor front. This was the DLL4 maturation index (MI) that ranged from 0.1 to 0.7 (median 0.34) (Figure 2d). There was no association between DLL4 MI and histopathological variables (Table 3).

DLL4 in the plasma

The concentration of DLL4 in the plasma ranged from 55-81pg/ml (mean 62.8) before and this dropped to 55-62 (mean 58.2) after hysterectomy (Figure 3). Paired analysis showed a significant p-value of 0.04. There was no association of plasma DLL4 with vascular parameters or cancer cell DLL4 expression (data not shown).

Table 3. Association between DLL4-maturation index (MI) and histopathological variables

	DLL4-MI in t1-area		
	Low	*High*	*p*
Grade			
1	11	8	0.68
2	4	5	
3	2	3	
Depth, %		0.73	
<50	13	13	
>50	4	3	
Endocervix		0,41	
No	13	14	
Yes	4	2	

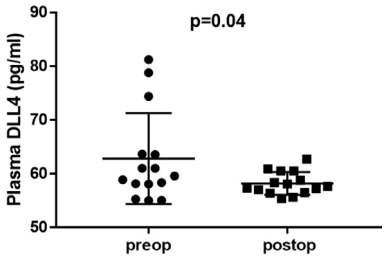

Figure 3. Pre- and post-operative plasma DLL4 levels in patients with stage I endometrial cancer.

Discussion

The Notch signaling pathway is a fundamental parameter in cell-to-cell communication system. Besides, it is involved in the coordination of pathway signaling that needs the regulation of gene mechanism controlling various procedures in cell differentiation. It regulates processes like embryogenesis, renewal of both tissues and cells, and organogenesis [7]. The Notch system is composed by four receptors (NOTCH 1, NOTCH 2, NOTCH 3, and NOTCH 4) and five ligands (Delta like ligand 1, 3, 4, Jagged 1, 2). Notch signaling components are expressed in tumor endothelial cells with DLL4 being the leading agent considering vasculogenesis. DLL4 is also markedly induced in tumor vessels [8-10].

Resent data has demonstrated that DLL4 is abundantly expressed in tumor endothelial cells of cancer vessels, especially compared to DLL4 expression of neighboring normal vessels [14,15]. Two endothelial cell types are essential for sprouting angiogenesis: the "tip" and the "stalk" cell [16]. As mentioned before, Notch signaling controls multiple aspects of endothelial cell function, such as growth, migration, lumen formation and arterial–venous determination [16,17]. Studies have revealed the role of DLL4 in regulating the specification of endothelial cells into tip and stalk cells during angiogenic sprouting [18-22]. A recent study reported that endothelial basement membrane can limit tip cell formation by inducing DLL4 signaling [23].

Analysis of Notch signaling revealed high DLL4 activity in stalk cells but low levels of Notch signaling in tip cells. Conversely, tip cells overexpress DLL4 that in filopodia-rich endothelial tip cells, lead to vasculogenesis and it is believed to activate Notch and suppress the tip phenotype in adjacent (stalk) endothelial calls. The key role of DLL4 in cancer angiogenesis can be revealed by the deletion of a single allele of DLL4 that results in tumor growth reduction due to defects in the vasculature formation [10,24]. Other studies reported that anti-DLL4 agents caused overgrowth of a faulty tumor vasculature that inhibited tumor growth [25,26]. Kontomanolis et al reported that DLL4 is overexpressed in breast cancer angiogenesis, while Koukourakis et al revealed that vascular DLL4 expression is associated with prognosis and radiotherapy response for head and neck cancer [27,28].

DLL4 mediated activation of the Notch signaling pathway results in reduced responsiveness of the endothelium to VEGF angiogenic signaling, allowing the prevalence of mature vessels [11]. We, therefore, examined the expression of DLL4 by tumor vessels and assessed the DLL4+ MVD and, subsequently, the vascular maturation index by dividing the DLL4+ with the CD31+ MVD in the invading tumor front and inner tumor areas. The percentage of DLL4+ vessels in the invading tumor front ranged from 10-70%, showing a wide difference in the quality of the neo-vasculature produced among endometrial tumors of the same histology and differentiation. Of interest, the DLL4 maturation index was further reduced in inner tumor areas, showing that DLL4 expression is likely to have an accessory rather than a decisive role in the survival of tumor vessels in inner tumor areas. Other pathways, like the angiopoietin or the fibroblast growth factor may be involved, which demands thorough investigation [12,13].

In addition, our study reported increased DLL4 expression by cancer cells. This result confirms the theory that DLL4 mediates carcinogenesis via vascular formation, while DLL4 overexpression by tumor cells may also be involved. Reports have proven a link between the expression profile of DLL4 from cancer cells as it can be a predictor of pelvic lymph node metastasis and a prognostic biomarker in patients with early-stage cervical, breast, lung, colorectal, brain and thyroid cancer with many studies investigating the specific prognostic application in clinical practice during the past decade [29-33]. Moreover, we report a DLL4 overexpression in the plasma that was associated with an important decrease postoperatively ($p<0.05$). Not many data has evaluated the pre and postoperative results of DLL4 expression in patients with cancer. Kontomanolis et al [28], suggested in a previous study DLL4 plasma levels measurement can reliably estimate the total DLL4 vasculature activity of the tumor, but more studies are needed to reach safe conclusions.

This pilot study suggests that DLL4 is overexpressed in endometrial cancer cells and tumor vasculature. DLL4 is also released in the plasma and its levels can be measured by ELISA. DLL4 plasma levels were notably decreased postoperatively. The percentage of DLL4+ vessels in the penetrating sample ranged from 10-70%, indicating a large difference in the quality of angiogenesis produced between the endometrial tumors of the same histological type and differentiation. The clinical relevance of the findings demands further evaluation.

Conflict of interests

The authors declare no conflict of interests.

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