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Vascular Biology, Atherosclerosis and Endothelium Biology

Expression of Vascular Notch Ligand Delta-Like 4 and Inflammatory Markers in Breast Cancer

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Delta-like ligand 4 (Dll4) is a Notch ligand that is predominantly expressed in the endothelium. Evidence from xenografts suggests that inhibiting Dll4 may overcome resistance to antivascular endothelial growth factor therapy. The aims of this study were to characterize the expression of Dll4 in breast cancer and assess whether it is associated with inflammatory markers and prognosis. We examined 296 breast adenocarcinomas and 38 ductal carcinoma *in situ* tissues that were represented in tissue microarrays. Additional whole sections representing 10 breast adenocarcinomas, 10 normal breast tissues, and 16 angiosarcomas were included. Immunohistochemistry was then performed by using validated antibodies against Dll4, CD68, CD14, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), CD123, neutrophil elastase, CD31, and carbonic anhydrase 9. Dll4 was selectively expressed by intratumoral endothelial cells in 73% to 100% of breast adenocarcinomas, 18% of *in situ* ductal carcinomas, and all lactating breast cases, but not normal nonlactating breast. High intensity of endothelial Dll4 expression was a statistically significant adverse prognostic factor in univariate ($P = 0.002$ and $P = 0.01$) and multivariate analyses ($P = 0.03$ and $P = 0.04$) of overall survival and relapse-free survival, respectively. Among the inflammatory markers, only CD68 and DC-SIGN were significant prognostic factors in univariate (but not multivariate) analyses of overall survival ($P = 0.01$ and 0.002, respectively). In summary, Dll4 was expressed by endothelium associated with breast cancer cells. In these retrospective subset analyses, endothelial Dll4 expression was a statistically significant multivariate prognostic factor. ***(Am J Pathol 2010, 176:2019–2028; DOI: 10.2353/ajpath.2010.090908)***

The growth of tumors requires angiogenesis,[1] which is the consequence of increased expression of proangiogenic factors (for example, vascular endothelial growth factor A [VEGF][2,3]). The expression of VEGF in cancer is controlled by oncogenic signaling,[4] hypoxia,[5] and inflammatory cells.[6] Although there is redundancy among proangiogenic factors in advanced cancer,[7] many *in vivo* early stage cancer models show VEGF dependence.[8,9]

This observation has been exploited clinically, where the addition of an anti-VEGF antibody (bevacizumab) to first line taxane-based chemotherapy in recurrent/metastatic breast cancer was associated with prolongation of progression free survival (from a median of 5.9 to 11.8 months, $P < 0.001$).[10] Nevertheless, there was no statistically significant overall survival benefit, and all patients in this trial eventually progressed after 4 years.[10] Furthermore, a trial evaluating the addition of bevacizumab to capecitabine in previously treated metastatic/advanced breast cancer demonstrated only a 10.7% improvement in response rate and no survival benefit.[11] To date, there are no validated clinical, radiological, or molecular biomarkers that can predict the survival benefit afforded by bevacizumab.[12–15] Clinical data suggest that antiangiogenic drugs are active in breast cancer,[10,16] and it may be necessary to identify biomarkers that predict their benefit.

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Additional agents that disrupt functional angiogenesis have been developed to target tumors resistant to anti-VEGF therapy.[17,18] Recent studies have focused on Delta-like ligand 4 (Dll4), a ligand for Notch receptors 1, 3, and 4[17–19] that is predominantly expressed by endothelial cells.[17–19] Transgenic mice in which Dll4 was replaced by a reporter gene showed that Dll4 expression is restricted to large arteries during development.[20,21] Furthermore, Dll4 heterozygous knockout mice are reported to have defective arterial development[22] and venous malformations.[22]

Experimental systems[17,23,24] have shown that Dll4-Notch inhibition leads to increased sprouting and branching of vessels in association with gradients of VEGF. Conversely, VEGF blockade causes a reduction in Dll4 expression and vessel sprouting.[17,18,23–27] In addition, endothelial cells transfected with Dll4 down-regulated VEGF receptors KDR and neuropilin1 and showed reduced proliferative and migratory responses to VEGF.[28] The implication of this research is that Dll4-Notch signaling regulates endothelial sprouting and branching to form functional vascular beds, under the control of VEGF and by autoregulation of VEGF signaling.[23]

Disruption of Dll4 signaling by overexpression or inhibition of Dll4 may impair angiogenesis,[17,18] and blockade of Dll4-Notch signaling results in an increased density of nonfunctional vasculature and is associated with a reduction in the growth of human tumor xenografts.[17,18] Indeed, certain xenografts that are resistant to anti-VEGF therapy are reported to be sensitive to anti-Dll4,[17,18,29] and combination treatment with anti-VEGF and anti-Dll4 has additive inhibitory effects on tumor growth.[18] Together these data provide a rationale to target Dll4 in cancer and suggest that Dll4 may have a role in mediating resistance to anti-VEGF therapies.

Besides direct vascular effects, Fung et al[30] showed that Dll4-Notch signaling in macrophages stimulates a proinflammatory response, which may be proangiogenic.[6] Moreover, Shojaei et al[31,32] have reported that bevacizumab resistance in certain preclinical *in vivo* cancer models is causally associated with tumor infiltration by myeloid cells.

The characterization of Dll4 protein expression in human cancer is important for the rational design of clinical trials to test the safety and activity of anti-Dll4 therapy. Defining the pattern of Dll4 expression, in association with markers of inflammation, may identify subgroups with distinct clinical behavior and responses to treatment. The aims of this study were to characterize the *in situ* expression of Dll4 in breast cancer, to assess the association between Dll4 and established markers of inflammation (CD68, CD14, neutrophil elastase, CD123, and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin [DC-SIGN]) and hypoxia (carbonic anhydrase 9 [CA9]), and to determine the prognostic significance of these markers.

Materials and Methods

Patients and Tissue Samples

Formalin-fixed paraffin-embedded (FFPE) tissues were obtained for 296 sequential patients with breast adenocarcinoma (surgery was performed between 1989 and 1998 at the John Radcliffe Hospital, Oxford, UK). Patients were treated with a wide local excision and postoperative radiotherapy or mastectomy with or without postoperative radiotherapy. Postoperative chemotherapy (600 mg/m^2 cyclophosphamide, 40 mg/m^2 methotrexate, and 600 mg/m^2 5-fluorouracil intravenously each on day 1 of a 21-day cycle $\times$6) and hormonal therapy (tamoxifen 20 mg daily for 5 years) were offered according to local protocols. Demographic, pathological, and treatment details are provided in Supplemental Table S1 (see *http://ajp.amjpathol.org*). Sample size was determined by the availability of tissue with clinicopathological data, survival follow-up, and ethical approval for research. Two cases had no survival data available. Follow-up data were correct as of January 2008, with a median follow-up time of 10 years, a median overall survival of 13.7 years, and a median relapse-free survival of 13.8 years. Estrogen receptor (ER) content was determined by using an enzyme-linked immunosorbent assay technique (Abbott Laboratories, Abbott Park, IL). Tumors were considered positive when cytosolic ER levels were $>$10 fmol/mg of total cytosolic protein. Receptor values were monitored by participation in the European Organization for Research and Treatment of Cancer (EORTC) quality control scheme. Human Epidermal growth factor Receptor 2 (HER2) status was assessed with the HercepTest (Dako, Carpinteria, CA).

Tissue microarrays (TMAs) were assembled as described previously[33] with three replicate cores for each tumor. Tissue from 38 patients with breast ductal carcinoma *in situ* was also represented in TMAs for analysis. An additional 10 breast adenocarcinomas, five normal breast resections, five normal lactating breast tissues, seven breast angiosarcomas, and nine nonbreast angiosarcomas (five skin, one duodenal, one liver, one pleural, and one vaginal) were also collected (John Radcliffe Hospital) to investigate the expression of Dll4 in whole sections.

Approval was obtained for the use of all human tissue from the local research ethics committee (C02.216). The National Cancer Institute's Reporting Recommendations for Tumor Marker Prognostic Studies criteria were used in the design, analysis, and interpretation of this research.[34]

In Situ *Hybridization*

A 727 base ^{35}S-labeled (^{35}S-UTP 800 Ci/mmol; PerkinElmer, Waltham, MA) antisense riboprobe 100% homologous to human DLL4 (position 2089 to 2815 of GenBank accession NM_019074.2) was generated by using T3 RNA polymerase (Promega, Southampton, UK) from a linearized blunt ended vector containing the above insert. Isotopic *in situ* hybridization, washes, and developing were performed by using previously described methods.[35] In brief, FFPE tissue sections were deparaffinized in xylenes and rehydrated through graded alcohols. Deproteination was performed for 15 minutes at 37°C with 20 μg/ml proteinase K (Sigma-Aldrich, Gillingham, UK). Slides were air-dried before hybridization overnight at 55°C. Posthybridization, a series of increasingly stringent washes were performed, and unhybridized probe was digested

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