

Heparanase and COX-2 Expression as Predictors of Lymph Node Metastasis in Large, High-grade Breast Tumors

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Abstract. *Background/Aim:* Heparanase (HPA) contributes to breast cancer metastasis by facilitating the breakdown of the basement membrane and extracellular matrix. High expression of HPA is thought to be associated with increased nodal involvement and poor survival in patients with breast cancer. Overexpression of cyclooxygenase-2 (COX-2) in breast cancer is associated with indicators of poor prognosis such as lymph node metastasis, poor differentiation, and large tumor size. The underlying mechanism by which HPA and COX-2 overexpression increases the metastatic potential of breast cancer is not fully-understood. To enhance our understanding over these mechanisms, we aimed to investigate the relationship between the size of the tumor and HPA expression, tumor grade as well as lymph node status in patients with breast cancer. *Materials and Methods:* Immunohistochemical analysis of HPA and COX-2 expression was performed on 246 breast tumor samples. The expression of HPA was correlated with COX-2 expression, tumor grade, lymph node status, oestrogen receptor status. *Results:* The overexpression of HPA and COX-2 was associated with increased likelihood of lymph node positivity in large, high-grade tumors. High-grade tumors with size greater than 20 mm, that overexpressed HPA, were 4-times

more likely to be associated with lymph node involvement (OR 4.71, CI 1.21-18.25). Whereas, tumors greater than 20 mm in size were 5-times more likely to metastasize to the regional lymph nodes, if associated with overexpression of COX-2 (OR 5.5, CI 1.2-24.8). *Conclusion:* Expression of HPA appears to be a key mechanism by which large, high-grade breast tumors metastasize to regional lymph nodes, while COX-2 overexpression may be an independent predictor of lymph node positivity.

Heparanase (HPA) is a β -endoglucuronidase enzyme that acts on heparan sulphate (1), the main polysaccharide component of the extracellular matrix, leading to the degradation of adhesion molecules and breakdown of the extracellular matrix and basement membrane (2) aiding invasion (3-5). It is suggested that HPA may have a role in facilitating both tumor cell invasion and neovascularization, two critical steps in tumor progression. In addition, HPA overexpression may lead to tumor growth and angiogenesis (6-10). It is suggested that destruction of the basal membrane and formation of new blood vessels may be the foundation for HPA involvement in tumor formation. Cyclooxygenase-2 (COX-2) is overexpressed in a variety of malignant tumors including breast cancer. Overexpression of COX-2 in breast cancer is associated with indicators of poor prognosis such as lymph node metastasis, poor differentiation, and large tumor size (11).

Axillary lymph node metastases remain the most important prognostic factor for breast cancer. If predictive accuracy of the metastatic potential of breast cancer can be enhanced, this will provide useful information for patients and healthcare professionals to make informed choices regarding local and systemic treatment planning. It remains an on-going challenge to identify biological factors that may enhance the

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predictability of lymph node metastasis of breast cancer. Larger size of the tumor, higher histological grade and young age are recognised predictors of axillary lymph node metastases. However, the underlying mechanism by which these biological variations affect the capacity of a breast tumor to spread to regional lymph nodes is not fully understood.

COX-2 and HPA can potentially provide additional information to identify patients at an increased risk of lymph node metastasis. The experimental studies in transgenic mice found that expression of HPA was associated with branching of ducts and alveolar development in mammary glands (12). Given the role of these molecules in cell invasion and angiogenesis, it is plausible that increased nodal metastasis in larger breast cancer is mediated by HPA and COX-2 expression. The aim of the present study was to investigate the relationship between the size of the tumour and HPA expression, tumour grade as well as lymph node status in patients with breast cancer.

Materials and Methods

Archived tissue samples from cases with and without nodal metastasis treated for breast cancer at a large Teaching Hospital in the UK, for the period between 1st January 2000 and 31st December 2004 were included in the study. The mean age at diagnosis in the study was 64 (range=30-90) years.

Immunohistochemical analysis of HPA and COX-2 expression was performed on 123 pairs (one with metastasis, one without) of breast tumor samples matched for age (± 1 year) and tumor size (± 1 mm). Immunohistochemistry for HPA was carried-out on the paraffin-embedded, formalin-fixed breast cancer tissue using the Bondmax (Vision Biosystems: Leica Biosystems Newcastle Ltd, Balliol Business Park West, Newcastle Upon Tyne, NE12 8EW United Kingdom) automated staining machine using standardised techniques. Antigen assessment was carried out on each case using the Heparanase antibody (HPA-1; Insight Laboratories, diluted 1:50 with Bondmax antibody diluent, Vision Biosystems: AR9352) and Cyclooxygenase-II antibody (COX-2; Abcam Laboratories; ABCAM, 330 Cambridge Science Park, Cambridge, CB4 0FL, UK, diluted 1:75 with Bondmax antibody diluent). Slides for HPA immunohistochemical analysis were examined microscopically and scored 0 for negative and 1 for positive according to visualisation of the brown chromagen precipitate. Slides for COX-2 immunohistochemical analysis were examined microscopically and each slide was scored on a scale of 0-3 according to the staining intensity of the reaction (0=negative; 1=weakly positive; 2=moderately positive; 3=strongly positive). Scoring was carried out by the chief investigator and an independent colleague. Results were compared and discrepancies were discussed and decided upon using a double-headed microscope.

Statistical analysis was performed on the completed dataset of 246 patients using PASW Statistics 18 software (SPSS Inc., Chicago, Illinois, USA). Lymph node (LN) status was explored using conditional binary logistic regression (when patients were matched on age (± 1 year) and tumour size (± 1 mm) variables). The 4 explanatory variables were Grade of Tumour, ER status, heparanase and COX-2 expression and selected 2nd order interactions. Backward elimination was used to obtain the

parsimonious solution. In order to further clarify the identified solution, cross-tabulations were used with the associated Chi-squared statistics. A p -value < 0.05 denoted the presence of a statistically significant difference.

Results

The mean age at diagnosis in the study was 64 (range 30-90) years. For patients with large tumors (> 20 mm), the conditional binary logistic regression analysis identified that patients with COX-2 overexpression were 5-times more likely to have a metastasis to the regional lymph nodes (OR 5.5, CI 1.2-24.8). Again, only for patients with large tumors (> 20 mm), the conditional binary logistic regression analysis identified that patients that were diagnosed with overexpressed HPA (heparanase-positive) and had high-grade tumors were nearly 5-times more likely to be associated with lymph node involvement (OR 4.71, CI 1.21-18.25). This interaction can be more clearly understood in Table I (Chi-square=6.29; $p=0.012$). Seventy-five percent of all high-grade tumors that had overexpressed HPA were associated with positive lymph nodes.

The above differences and associations were retained in the model when a multivariable analysis was performed using backward elimination.

Discussion

The present study demonstrates the novel finding that in our cohort of breast cancer patients, overexpression of HPA in high-grade large tumors was associated with increased incidence of regional lymph node metastases. This suggests that HPA expression is a key mechanism in the metastasis of high grade breast cancer. In breast tumors, that did not over-express HPA, we found no association between non-high tumors and lymph node status or between high grade tumors and lymph node status.

It is well-recognised that tumor histological grade can be associated with metastasis and poor disease outcome (13). HPA has established proliferative, invasive and metastatic properties (14). Previous studies have suggested that HPA expression in breast cancer tumors is tumor size- (10) or ER-dependent (15) and HPA expression is also positively correlated with breast cancer tumor grade (16). Our data add to the field by suggesting that poorly-differentiated tumors use increased HPA expression as a key mechanism to initiate or propagate metastasis to regional lymph nodes.

As the association between overexpression of the HPA and lymph node metastases was only found in large, high-grade tumors, it is postulated that high grade tumors are more capable of expressing and secreting HPA which in turn may facilitate cell proliferation and metastasis, leading to an increased ability to spread to distant sites. Evidence from transgenic animal experiments, whereby

Table I. Lymph node positivity by Heparanase overexpression (positive vs. negative) and tumour grade (high grade vs. non-high grade).

Grade		Non-high grade	High grade
Heparanase	Negative	50.0% (25/50)	42.5% (17/40)
	Positive	43.8% (21/48)	75.0% (18/24)

Values are percentages with positive lymph node status (numbers with positive lymph node status/total numbers).

overexpression of HPA in tumors of low metastatic potential cells conferred a high metastatic potential, also supports this hypothesis (17).

Our study is in concordance with what is reported in the literature, that HPA is a potent tumor modulator due to its pro-tumorigenic, pro-angiogenic, and pro-metastatic activities (18). However, we found that this association was limited to large, high-grade tumors. The differences in patterns of progression of various grades and types of breast cancer is a well-recognized phenomenon (19); our data add to the current understanding by showing that in high-grade tumors HPA overexpression contributes to nodal metastases. There is always a possibility of a Type I error due to small numbers, however our results are supported by clinical experience. In clinical practice, high-grade tumors are more likely to be associated with lymph node positivity (20). The hypothesis generated by a study of positive sentinel nodes that HPA may be involved in lymph node positive tumors (21) is supported by our data which demonstrate that HPA expression in high grade breast cancer may lead to lymph node positivity (4, 15, 22).

Past studies have demonstrated that there is a significant relationship between HPA, COX-2 and angiogenesis. The present study does not reveal a correlation between HPA expression and COX-2 expression (data not shown). The present study, however, supports previous studies which demonstrate that COX-2 is not correlated with ER status (23).

In conclusion, the present study has shown that expression of HPA appears to be a key mechanism by which large, high-grade breast tumors metastasize to regional lymph nodes, while COX-2 overexpression is an independent predictor of lymph node positivity.

References

- Nakajima M, Irimura T, Di Ferrante N and Nicolson GL: Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. *J Biol Chem* 259(4): 2283-2290, 1984.
- Wang Z, Xu H, Jiang L, Zhou X, Lu C and Zhang X: Positive association of heparanase expression with tumor invasion and lymphatic metastasis in gastric carcinoma. *Mod Pathol* 18(2): 205-211, 2005.
- Ilan N, Elkin M and Vlodavsky I: Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol* 38(12): 2018-2039, 2006.
- Parish CR, Freeman C and Hulett MD: Heparanase: a key enzyme involved in cell invasion. *Biochim Biophys Acta* 1471(3): M99-108, 2001.
- Rickles FR: If heparanase is the answer, what is the question? *J Thromb Haemost* 4(3): 557-559, 2006.
- Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L and Pecker I: Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat Med* 5(7): 793-802, 1999.
- Vlodavsky I, Goldshmidt O, Zcharia E, Atzmon R, Rangini-Guatta Z, Elkin M, Peretz T and Friedmann Y: Mammalian heparanase: involvement in cancer metastasis, angiogenesis and normal development. *Semin Cancer Biol* 12(2): 121-129, 2002.
- Costa C, Soares R, Reis-Filho JS, Leitao D, Amendoeira I and Schmitt FC: Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. *J Clin Pathol* 55(6): 429-434, 2002.
- Imada T, Matsuoka J, Nobuhisa T, Okawa T, Murata T, Tabuchi Y, Shirakawa Y, Ohara N, Gunduz M, Nagatsuka H, Umeoka T, Yamamoto Y, Nakajima M, Tanaka N and Naomoto Y: COX-2 induction by heparanase in the progression of breast cancer. *Int J Mol Med* 17(2): 221-228, 2006.
- Maxhimer JB, Quiros RM, Stewart R, Dowlatshahi K, Gattuso P, Fan M, Prinz RA and Xu X: Heparanase-1 expression is associated with the metastatic potential of breast cancer. *Surgery* 132(2): 326-333, 2002.
- Denkert C, Winzer KJ and Hauptmann S: Prognostic impact of cyclooxygenase-2 in breast cancer. *Clin Breast Cancer* 4(6): 428-433, 2004.
- Subbaramaiah K, Chung WJ and Dannenberg AJ: Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. *J Biol Chem* 273(49): 32943-32949, 1998.
- Porter GJ, Evans AJ, Pinder SE, James JJ, Cornford EC, Burrell HC, Chan SY, Cheung KL and Robertson JF: Patterns of metastatic breast carcinoma: influence of tumour histological grade. *Clin Radiol* 59(12): 1094-1098, 2004.
- Gotte M and Yip GW: Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. *Cancer Res* 66(21): 10233-10237, 2006.
- Elkin M, Cohen I, Zcharia E, Orgel A, Guatta-Rangini Z, Peretz T, Vlodavsky I and Kleinman HK: Regulation of heparanase gene expression by estrogen in breast cancer. *Cancer Res* 63(24): 8821-8826, 2003.
- Liu ZZ, Zhang HW, Wei B and Cui SD: Correlation of expression of heparanase to angiogenesis and prognosis of breast cancer. *Ai Zheng* 23(11): 1342-1345, 2004.
- Zcharia E, Metzger S, Chajek-Shaul T, Friedmann Y, Pappo O, Aviv A, Elkin M, Pecker I, Peretz T and Vlodavsky I: Molecular properties and involvement of heparanase in cancer progression and mammary gland morphogenesis. *J Mammary Gland Biol Neoplasia* 6(3): 311-22, 2001.
- Gomes AM, Stelling MP and Pavao MS: Heparan sulfate and heparanase as modulators of breast cancer progression. *Biomed Res Int* 2013: 852093, 2013.

- 19 Shackney SE and Silverman JF: Molecular evolutionary patterns in breast cancer. *Adv Anat Pathol* 10(5): 278-290, 2003.
- 20 Barth RJ Jr., Danforth DN Jr., Venzon DJ, Straus KL, d'Angelo T, Merino MJ and Gerber L: Level of axillary involvement by lymph node metastases from breast cancer is not an independent predictor of survival. *Arch Surg* 126(5): 574-577, 1991.
- 21 Cohen I, Maly B, Simon I, Meirovitz A, Pikarsky E, Zcharia E, Peretz T, Vlodaysky I and Elkin M: Tamoxifen induces heparanase expression in estrogen receptor-positive breast cancer. *Clin Cancer Res* 13(14): 4069-4077, 2007.
- 22 Barth A, Craig PH and Silverstein MJ: Predictors of axillary lymph node metastases in patients with T1 breast carcinoma. *Cancer* 79(10): 1918-1922, 1997.
- 23 Sun L, Yu DH, Sun SY, Zhuo SC, Cao SS and Wei L: Expressions of ER, PR, HER-2, COX-2, and VEGF in primary and relapsed/metastatic breast cancers. *Cell Biochem Biophys* 68(3): 511-516, 2014.

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