Journals Club

Review of:
Demethylation of urokinase promoter as a prognostic marker in patients with breast carcinoma

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Abstract of the original article

Purpose: Urokinase (uPA) is expressed in a number of highly invasive malignancies including breast cancer. Because production of uPA is associated with breast cancer progression and can serve as a useful prognostic marker, the purpose of this study was to examine the role of uPA promoter methylation as an indicator of uPA production in breast cancer patients.

Experimental design: We examined the methylation status of the uPA promoter and the levels of uPA expression in normal human breast epithelial cells and several human breast cancer cells by bisulfite sequencing analysis and reverse transcription-PCR. We also analyzed the methylation status of the uPA promoter in surgical biopsy samples from patients with breast cancer of different grades, as determined by the Elston–Ellis histological grading system.

Results: Expression of uPA mRNA was only detected in the highly invasive estrogen receptor-negative breast cancer cell lines, where the promoter was completely demethylated. In normal and low invasive breast cancer cells, the uPA promoter was methylated, resulting in lack of uPA mRNA expression. Analysis of biopsy samples showed that demethylation of the uPA promoter is associated with malignant transformation. Reverse transcription-PCR analysis revealed that this demethylation of the uPA promoter is directly associated with induction of uPA mRNA expression, which is well known to be associated with poor prognosis in breast cancer patients.

Conclusions: This study indicated that uPA expression in breast cancer patients is under epigenetic control via methylation of its promoter. Determination of uPA promoter methylation can therefore serve as an early reliable indicator of uPA production in breast cancer patients.

Review

The capacity of tumour cells to metastasise is considered the primary cause of breast cancer mortality.

Numerous observations have indicated that the matrix remodelling urokinase system is vital in distant spread of breast cancer. Key to this is the finding that a high level of the urokinase-type plasminogen activator (uPA) in primary breast tumour tissue is a strong indicator of poor prognosis in patients afflicted with breast cancer [1,2]. The urokinase system, including uPA and its plasminogen activator inhibitor-1 (PAI-1), has now reached the highest level of evidence (LOE-1) regarding its clinical relevance

and can be considered an approved prognostic marker for breast cancer progression [3]. Hence, it is not surprising that a synthetic inhibitor to uPA, WX-UK1, has entered clinical trials in patients with advanced cancer [4,5].

Promoter DNA methylation is a booming field in cancer biology [6] since it has become apparent that this epigenetic type of gene regulation contributes to many aspects of cancer biology such as tumour initiation, tumour aggressiveness and the tumour's behaviour during endocrine and chemotherapy [7]. DNA methylation is an epigenetic alteration of the genetic information that is involved in gene silencing [8]. It occurs only on cytosines of cytosine–guanine dinucleotides (CpGs). In normal cells, generally only repetitive and intergenic genomic regions are methylated while regulatory regions, except for a limited number of tissue-specific genes, are not [9–11]. In cancer, DNA methylation shifts towards the regulatory regions of genes resulting in silencing of tumour suppressor genes [12,13]. In addition, the overall genome is destabilized due to global DNA hypomethylation [14]. Several properties make epigenetic changes at the level of the DNA attractive as diagnostic targets. First, DNA methylation is very stable, even allowing detection in poorly conserved clinical material. Second, the DNA methylation tag can be amplified allowing sensitive and quantitative detection. Finally, detection of DNA methylation is applicable to fixed, paraffin-embedded material allowing rapid transfer of a diagnostic test to clinical routine. Although DNA methylation markers have not found their way into the clinic yet, they could be important and powerful diagnostic, prognostic or predictive markers not far ahead.

In a series of interesting papers, the research group led by Prof Rabbani has explored the importance of uPA promoter DNA methylation in breast cancer proposing it possesses prognostic significance. Their initial preliminary work [15] showed that the uPA promoter is methylated in non-invasive breast cancer cell lines (MCF-7 and T-47D) that do not express uPA messenger RNA (mRNA), while the uPA promoter was not methylated in the highly invasive MDA-MB-231 cell line that does express uPA mRNA. We have replicated these results [16]. Furthermore, the work showed that uPA mRNA silencing was 5-azacytidine sensitive indicating that promoter DNA methylation was causally involved. Recently, the initial experiments of Xing *et al*. (1999) were extended to clinical breast tumour tissue samples [15]. In this study, over 30 CpG sites around the transcription start site of the uPA gene were analysed by bisulphite sequencing. Methylation of at least one CpG was found in 20 tumours, whereas 23 samples were found to be completely unmethylated. Notably, the

observed pattern was quite heterogeneous, and none of the CpGs was consistently methylated in the majority of the tumours. Of all CpG positions analysed, the percentage of CpG positions that showed methylation varied from 0% to 6.8%. This percentage is surprisingly low but may be an underestimation since the DNA used in the study was isolated from total tissue and not from micro-dissected tumour cells or stromal cells. Urokinase promoter DNA methylation in these breast cancer specimens was furthermore strongly negatively associated with tumour grade and mainly present in oestrogen receptor (ER)-positive tumours. Since ER-positive tumours represent a subgroup of tumours that is probably derived from the luminal epithelial cell lineage [18], the finding of Pakneshan *et al*. [17] might imply that the uPA promoter is methylated in this cell lineage. In a similar study, Chen *et al*. [19] detected uPA promoter methylation in 28% of the 93 primary breast cancer specimens was analysed. Due to the difference in approach the results of these two studies cannot be directly compared but both suggest a not-to-be-ignored role for uPA promoter DNA methylation in breast cancer. The consistent observation that DNA methylation of the uPA promoter is present in ER-positive tumours in both studies reassures this. Although not directly relevant to this short review but potentially relevant from a therapeutic perspective, Rabbani's group reported in a third paper on this subject that hypermethylation of the uPA promoter, leading to loss of uPA expression, can be induced by the pharmacological agent S-adenosyl-L-methionine [20].

Although it has been well established that uPA is an important prognostic marker [1,2] and DNA methylation of its promoter occurs in breast cancer, it is not established yet that the DNA methylation status of the urokinase promoter properly reflects uPA protein expression. The work from Rabbani and co-workers shows that promoter DNA methylation is an existing way to control uPA gene expression in breast cancer. The mRNA for uPA tends to be expressed only in those tumour samples which do not show methylation. However, not all tumours with a completely unmethylated copy of uPA express uPA mRNA, indicating additional regulatory mechanisms of uPA expression. Several transcription factor-binding sites (i.e. PAE-3/ETS, AP-1, NF-κB, and RelA) are present in the human urokinase promoter [21,22]. Some of them (ETS-1) also have prognostic impact and might be directly involved in uPA regulation [23]. In addition, growth regulatory factors (insulin growth factor-1, IGF-1) [24], heregulin [25], epidermal and fibroblast growth factors (EGFs and FGFs, respectively) [26], oestrogens [27], and integrins [28] regulate uPA expression in breast cancer cells *in vitro*. However,

none of these factors have been causally linked to uPA expression in human breast cancer. In addition, post-transcriptional regulation of uPA has been reported [29], but again its clinical significance remains a matter of debate. Another issue not to be ignored is the stromal contribution to urokinase production. Studies have shown that not only breast cancer cells themselves but also the adjacent stromal cells, and particularly myofibroblasts, produce uPA [30]. The significance of stromal uPA is underlined in a mouse model that is deficient for uPA since in these mice the growth of transplanted human tumours is significantly impaired [31]. In addition, a small study by us [16], suggests that DNA methylation of the uPA promoter does not contribute to the regulation of uPA expression in tumour-associated breast fibroblasts, while several cytokines, growth factors as well as cellular aging were capable of regulating stromal uPA expression [16,32].

Concluding, the fact that DNA methylation is involved in controlling uPA expression is an intriguing finding that justifies to be pursued in a more extensive series of primary breast cancer specimens with homogeneous treatment and well-documented clinical follow-up. In addition, the concrete functional consequences of DNA methylation of individual sites and regions in the uPA promoter regarding gene expression also require further studies. It remains to be shown if the promising results of the small series of clinical samples studied so far can be extended, and whether, ultimately, the prognostic impact of uPA promoter DNA methylation is as powerful as that of the uPA protein itself.

References

1. Jänicke F, Prechtl A, Thomssen C, *et al*. Randomized adjuvant chemotherapy trial in high risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. *J Natl Cancer Inst* 2001; **93**: 913–920.

2. Look MP, van Putten WLJ, Duffy MJ, *et al*. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. *J Natl Cancer Inst* 2002; **94**: 116–128.

3. Duffy MJ. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies. *Clin Chem* 2002; **48**: 1194–1197.

4. http://www.medicalnewstoday.com/index.php?newsid=8684

5. Muehlenweg B, Sperl S, Magdolen V, *et al*. Interference with the urokinase plasminogen activator system: a promising therapy concept for solid tumours. *Expert Opin Biol Ther* 2001; **1**: 683–691.

6. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; **1**: 163–167.

7. Maier S, Harbeck N, Schmitt M, *et al*. DNA methylation in breast cancer as diagnostic markers and targets for cancer therapy. In: Ross JS, Hortobagyi GN (Eds). *Molecular Oncology of Breast Cancer*. Sudbury, MA: Jones and Bartlett, 2004: 422–438.

8. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415–428.

9. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983; **301**: 89–92.

10. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* 1985; **228**: 187–190.

11. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *New Engl J Med* 2003; **349**: 2042–2054.

12. Esteller M, Fraga MF, Paz MF, *et al*. Cancer epigenetics and methylation. *Science* 2002; **297**: 1807–1808.

13. Esteller M. Epigenetic lesions causing genetic lesions in human cancer promoter hypermethylation of DNA repair genes. *Eur J Cancer* 2000; **36**: 2294–2300.

14. Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene* 2002; **21**: 5400–5413.

15. Xing RH, Rabbani SA. Transcriptional regulation of urokinase (uPA) gene expression in breast cancer cells: role of DNA methylation. *Int J Cancer* 1999; **81**: 443–450.

16. Martens JW, Sieuwerts AM, Vries JB, *et al*. Aging of stromal-derived human breast fibroblasts might contribute to breast cancer progression. *Thromb Haemost* 2003; **89**: 393–404.

17. Pakneshan P, Tetu B, Rabbani SA. Demethylation of urokinase promoter as a prognostic marker in patients with breast carcinoma. *Clin Cancer Res* 2004; **10**: 3035–3041.

18. Perou CM, Sorlie T, Eisen MB, *et al*. Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–752.

19. Chen CM, Chen HL, Hsiau TH, *et al*. Methylation target array for rapid analysis of CpG island hypermethylation in multiple tissue genomes. *Am J Pathol* 2003; **163**: 37–45.

20. Pakneshan P, Szyf M, Farias-Eisner R, Rabbani SA. Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. *J Biol Chem* 2004; **279**: 31735–31744.

21. Nerlov C, Rorth P, Blasi F, Johnsen M. Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. *Oncogene* 1991; **6**: 1583–1592.

22. Hansen SK, Nerlov C, Zabel U, *et al*. A novel complex between the p65 subunit of NF-kappa B and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *Embo J* 1992; **11**: 205–213.

23. Span PN, Manders P, Heuvel JJ, *et al*. Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer. *Oncogene* 2002; **21**: 8506–8509.

24. Dunn SE, Torres JV, Nihei N, Barrett JC. The insulin-like growth factor-1 elevates urokinase-type plasminogen activator-1 in human breast cancer cells: a new avenue for breast cancer therapy. *Mol Carcinogen* 2000; **27**: 10–17.

25. Mazumdar A, Adam L, Boyd D, Kumar R. Heregulin regulation of urokinase plasminogen activator and its receptor: human breast epithelial cell invasion. *Cancer Res* 2001; **61**: 400–405.

26. Boilly B, Vercoutter-Edouart AS, Hondermarck H, *et al*. FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev* 2000; **11**: 295–302.

27. Levenson AS, Kwaan HC, Svoboda KM, *et al*. Oestradiol regulation of the components of the plasminogen–plasmin system in MDA-MB-231 human breast cancer cells stably expressing the oestrogen receptor. *Br J Cancer* 1998; **78**: 88–95.

28. Chen J, Baskerville C, Han Q, *et al*. Alpha(v) integrin, p38 mitogen-activated protein kinase, and urokinase plasminogen activator are functionally linked in invasive breast cancer cells. *J Biol Chem* 2001; **276**: 47901–47905.

29. Montero L, Nagamine Y. Regulation by p38 mitogen-activated protein kinase of adenylate- and uridylate-rich element-mediated urokinase-type plasminogen activator (uPA) messenger RNA stability and uPA-dependent *in vitro* cell invasion. *Cancer Res* 1999; **59**: 5286–5293.

30. Nielsen BS, Sehested M, Duun S, *et al*. Urokinase plasminogen activator is localized in stromal cells in ductal breast cancer. *Lab Invest* 2001; **81**: 1485–1501.

31. Frandsen TL, Holst-Hansen C, Nielsen BS, *et al*. Direct evidence of the importance of stromal urokinase plasminogen activator (uPA) in the growth of an experimental human breast cancer using a combined uPA gene-disrupted and immunodeficient xenograft model. *Cancer Res* 2001; **61**: 532–537.

32. Sieuwerts AM, Klijn JGM, Henzen-Logmans SC, Foekens JA. Cytokine-regulated urokinase-type-plasminogen-activator (uPA) production by human breast fibroblasts *in vitro*. *Breast Cancer Res Treat* 1999; **55**: 9–20.