Hypoxia inducible factor-1α (HIF-1α) and matrix metalloproteinase 9 (MMP-9) expression in invasive breast cancer

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Abstract

Aim: Hypoxia has a key role in cancer progression and metastasis. Low tissue oxygen availability induces the activity of HIFs (Hypoxia-Inducible Factors) to up-regulate a panel of invasion related genes, in a plethora of solid tumors. MMPs (matrix metalloproteinases) are enzymes produced from normal and cancerous cells with the ability to degrade extracellular stroma, priming invasion. The aim of this study was to identify possible gene and protein associations between HIF-1α and MMP-9 in invasive breast cancer.

Materials and Methods: A total of 96 FFPE (Formalin-Fixed Paraffin-Embedded) breast cancer samples were evaluated for HIF-1α and MMP-9 protein expression. Publicly available gene expression datasets for breast cancer were analyzed for HIF-1a and MMP-9 gene expression. Results were correlated with clinicopathological parameters and patient clinical outcome.

Results: MMP-9 and HIF-1α showed a positive correlation both at the mRNA ($p<0.001$) and protein ($p=0.01$) expression levels, in samples from breast cancer patients. This correlation was specific because HIF-2b, another member of the HIF family, presented with a negative correlation with MMP-9 gene expression. Furthermore, mRNA and protein expression of MMP-9 had a negative correlation ($p<0.01$) with relapse-free survival (RFS) and a positive correlation ($p<0.01$) with lymph node status (N1), respectively. In addition, patients with high mRNA expression of HIF-1α had significantly ($p<0.001$) shorter OS and RFS than did patients with low levels of HIF-1a.

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Conclusions: HIF-1α and MMP-9 co-expression may represent a novel marker of worse clinical outcome in breast cancer patients, predicting increased metastatic capacity of cancer cells.

Introduction

Hypoxia is a major driving force of tumor progression and cancer metastasis [1]. The presence of tumor hypoxia is correlated with poor overall survival and increased metastatic incidence in patients with various solid tumor types, including breast cancer [2,3,4,5]. Hypoxic cancer cells tend to physically move toward an oxygen rich environment, being selected for higher invasiveness and metastatic capacity, promoting malignant progression [6]. The molecular sensor of cellular hypoxia is a family of transcription factors (Hypoxia Inducible Factors, HIFs) responsive to reduced intracellular oxygen availability. Hypoxia inhibits HIF degradation and promotes their transcriptional activity. Over 1000 HIF target genes have been identified so far, and amongst them HIF-1α regulates the expression of several key tumor progression genes (e.g CXCR4, RIOK3, LOX) [7,8]. Immunohistochemical studies on breast tumor samples have linked increased HIF-1α protein levels with increased risk of metastasis and mortality in unselected breast cancer patients [9,10,11].

In more detail, hypoxia-induced invasion and metastasis involves several steps where cancer cells must cross numerous extracellular matrix (ECM) barriers, such as the epithelial basement membrane and blood vessels or lymphatics. MMPs (matrix metalloproteinases) are a large family of metal-binding proteinases with enzymatic activity against all components of the ECM and basement membranes. MMP-9 has the ability of degrading extracellular matrix and directly regulates angiogenesis by increasing the bioavailability of the pro-angiogenic factor VEGF. In addition, the formation of experimental lung metastases in mice is reduced by downregulation of MMP-9 in cancer cells [12], and is also reduced in the MMP-2- and MMP-9-null mice as compared with wild-type mice [13].

However, there are limited data concerning possible molecular interactions between the two proteins in breast cancer progression. In the present study we investigated HIF-1α and MMP-9 immunohistochemical expression in invasive breast cancer tissue samples and its correlation with tumor clinicopathological parameters. Furthermore, we analyzed publicly available mRNA expression datasets for possible HIF-1a and MMP-9 gene associations.

Material and Methods

Tissue specimens

Formalin-fixed paraffin-embedded postsurgical specimens from human breast carcinomas were retrieved from the archives of the Department of Pathology, University Hospital of Patras, Greece. There were 96 FFPE (formalin fixed paraffin embedded) samples of invasive breast cancer. Data for breast cancer prognostic factors (HER2, proliferation index Ki67 and p53 protein) and receptor expression (ER, PR) were collected from the original pathology reports (Figure 1.A). This study was conducted according to the principles laid down by the Declaration of Helsinki.

Clinicopathological characteristics.		
Mean age at diagnosis (years)	56	Range: 32 – 80
Median tumor size (cm)	2.8	Range: 0.1 – 9.0
TNM stage n (%)	I IIa IIb IIIa IIIb IIIc IV	13(13.5) 18(18.8) 24(25) 8(8.3) 4(4.2) 12(12.5) 17(17.7)
Lymph node status, n (%)	Positive Negative	64/96 (66.7) 32/96 (33.3)
Tumour grade, n (%)	1 2 3	9/96 (9.4) 63/96 (65.6) 24/96 (24)
ER status, *n* (%)*	Positive Negative	74/96 (77.1) 22/96 (22.9)
PR status, *n* (%)*	Positive Negative	66/96 (68.8) 30/96 (31.3)
hER2 / c-erbB2 status, *n* (%)*	Positive Negative	31/96 (32.3) 65/96 (67.7)
p53 status, *n* (%)*	Positive Negative	47/96 (49) 49/96 (51)
Ki67 status, *n* (%)*	Low Intermediate High	57/96 (59.4%) 18/96 (18.8%) 21/96 (21.9%)

Figure 1.A Clinicopathological parameters of the in-house breast cancer patient cohort

Immunohistochemical evaluation

Immunohistochemical analysis was carried out as previously described [14]. Briefly, the anti-MMP-9 (Novocastra) or the anti-HIF-1α (R&D Systems) primary antibodies (dilutions 1:15 and 1:30, respectively) and Envision Detection System (DAKO, Hamburg, Germany) were used for visualization. For each section an assessment was made both of staining intensity and of percentage of cells staining in separate scales from 1 to 3. Then for each section the two scores were added to obtain the scores as 0, 1, 2, 3, 4, 5 or 6. Tumors having a final staining score of 1, 2 or 3 were lumped to a low expression group and 4, 5 or 6 to a high expression group.

Gene expression data and statistical analysis

Gene expression datasets were downloaded from The Cancer Genome Atlas (TCGA) portal (cancergenome.nih.gov/). Breast cancer survival analysis was performed through KM-plotter as described in Gyorffy et al. [15]. Statistical analysis was performed with SPSS, v15.0 for Windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

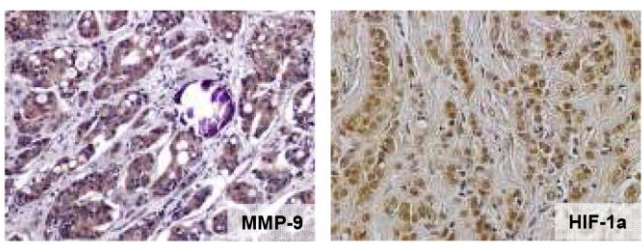

Figure 1.B Expression of MMP-9 and HIF-1α in breast cancer cells at 40x magnification

Results

1. Immunoexpression of HIF-1α and MMP-9 in breast carcinomas and adjacent normal tissue.

Immunohistochemical staining for HIF-1α and MMP-9 was performed on 96 and 95 tumors and uninvolved adjacent breast tissue, respectively. The normal breast tissue did not express either of the molecules. In breast cancer tissue, MMP-9 showed high cytoplasmic expression in 64/96 (66.6%) of cases, low in 22/96 (22.9%) and negative in 10/96 (10.5%) (Figure 1.B). There was also rare weak expression in endothelial cells and fibroblasts of cancer stroma. Positive HIF-1α expression was identified in cancer cells, while cancer stroma was negative (Figure 1.B). High expression of HIF-1α was found in 64/96 (66.6%) of cases, low in 18/96 (18.8%) and negative in 14/96 (14.6%). Importantly, there was a statistically significant correlation between expression of HIF-1α and MMP-9 in cancer cells ($r=0.264$, $p=0.01$). To further test a possible co-expression of HIF-1α and MMP-9 in breast cancer

at the mRNA level, we employed TCGA publicly available data from breast cancer patients to evaluate a potential correlation between HIF-1a and MMP-9 gene expression. We analyzed mRNA expression (RNA Seq V2 RSEM) data for HIF-1a and MMP-9 genes from an unselected cohort of 971 breast cancer patients with available data for both genes under study. HIF-1a mRNA showed a statistically significant positive correlation ($p<0.001$) with the mRNA expression of MMP-9, while there was a negative correlation between HIF-2b, another member of the hypoxia-inducible factor family and MMP-9 (Figure 1.C, upper panel). In addition, HIF-1a expression correlated positively with MMP-9 in estrogen receptor positive (ER+) cases ($p<0.01$), but not with ER- cases ($p=0.296$) from the same TCGA breast cancer patient cohort (Figure 1.C, lower panel and data not shown).

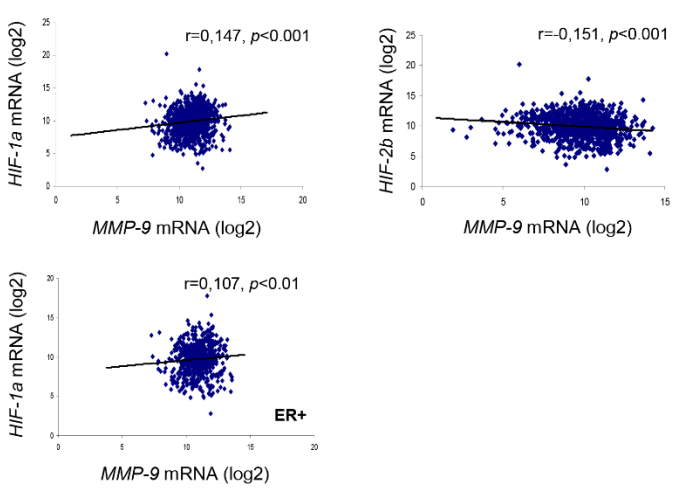

Figure 1.C HIF-1α and MMP-9 co-expression at the mRNA level (upper left panel), HIF-1β and MMP-9 co-expression at the mRNA level (upper right panel) and HIF-1β and MMP-9 co-expression at the mRNA level in ER+ breast cancer patients (lower left panel)

2. Association of HIF-1α and MMP-9 with clinicopathological data and clinical outcome.

In order to examine the correlation between expression patterns of MMP-9 and HIF-1α with clinicopathological parameters, statistical analysis was performed on both in-house cases with IHC expression data and publicly available datasets of breast cancer patients. The results showed that MMP-9 IHC expression correlated with a marginal significance ($p=0.046$) with the TNM stage of breast tumors and lymph node status ($p<0.01$). To assess in an extended cohort, possible correlation of HIF-1α and MMP-9 with breast cancer patient clinical outcomes, we employed microarray data of breast tumors from 4,142 patients [15]. 3,554 patients (all breast tumor types with valid probe sets) were analyzed for relapse-free survival (RFS) in conjunction to HIF1-a and MMP-9 gene expression. Those with high levels of HIF1-a and MMP-9 mRNA expression (the median value was used as the cutoff for low and high expression) had significantly shorter relapse-free survival than did patients with low levels of HIF-1a ($p<0.001$) and MMP-9 ($p<0.01$) (Figure 2.A and B, respectively). In addition, in the same dataset HIF1-a expression had an inverse correlation ($p<0.001$) with overall patient survival (OS) (Figure 2.C), while MMP-9 expression was inversely correlated ($p<0.01$) with OS in the estrogen receptor negative (ER-) group of patients (n=293) (Figure 2.D).

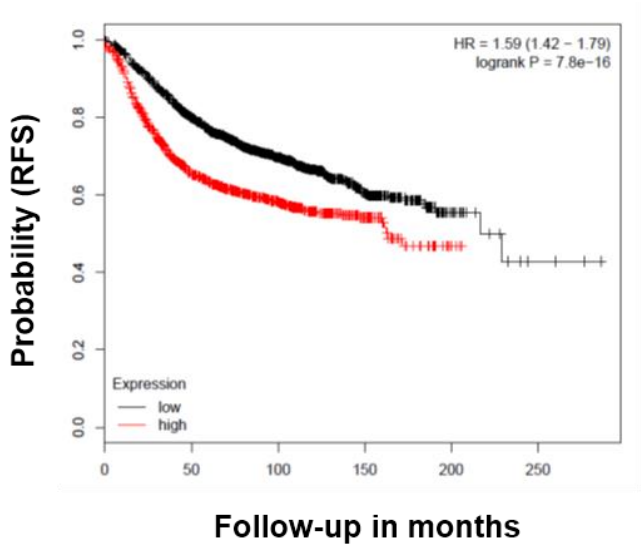

Figure 2.A Kaplan-Meier curves of relapse-free survival (RFS) for HIF-1a gene expression. Statistical significance was determined by log-rank test.

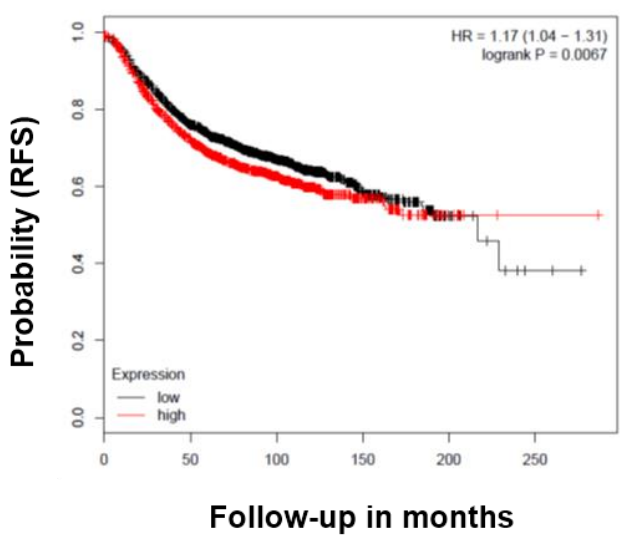

Figure 2.B Kaplan-Meier curves of relapse-free survival (RFS) for MMP-9 *gene expression. Statistical significance was determined by log-rank test.*

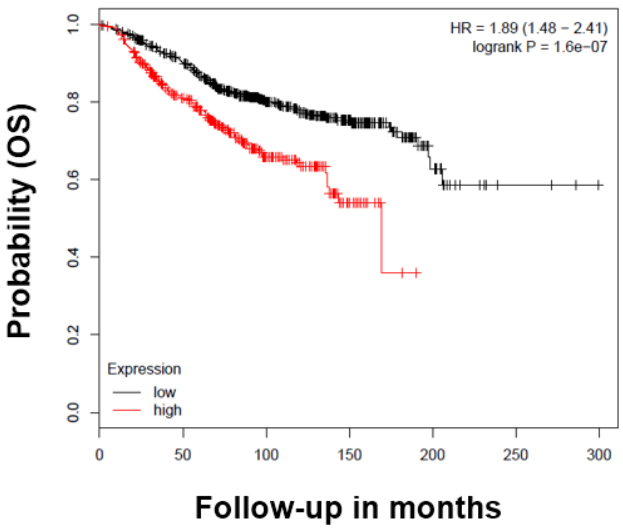

Figure 2.C Overall survival (OS) for HIF-1a in the whole patient cohort. Statistical significance was determined by log-rank test.

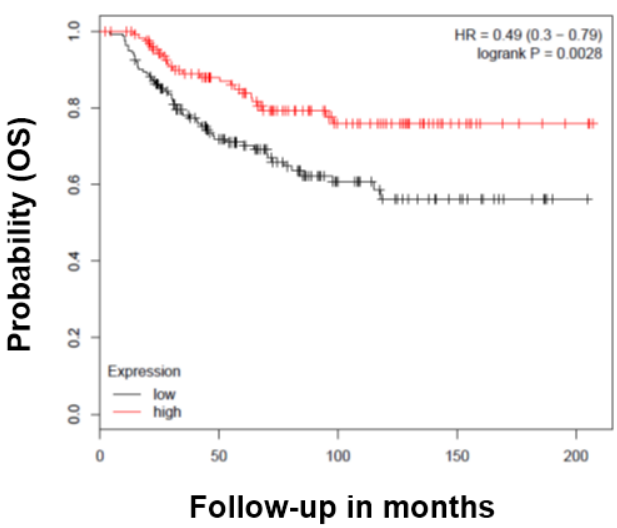

Figure 2.D Overall survival (OS) for MMP-9 in ER- breast cancer patients.

Discussion

The crucial role of hypoxia in tumor pathophysiology and metastasis is well documented [1]. However, the underlying mechanisms are still not fully elucidated. The metastatic cascade involves migration, invasion,

adhesion and survival of tumor cells and these cellular "states" constitute rate limiting steps in the developing metastases [4]. Hypoxia induces the invasive capacity of tumor cells, and through the activation of hypoxia-inducible transcription factors, a panel of invasion-promoting genes is upregulated. Important barrier to cell movement is the extracellular matrix which can be degraded by the enzymatic activity of matrix metalloproteinases, produced by cancer cells. MMP activity is a well-established hallmark of invasive phenotypes in many solid tumors and in breast cancer.

In the present study, MMP-9 protein expression was positively correlated with lymph node metastases, while high mRNA expression was correlated with shorter RFS and reduced OS in ER negative patients. At the same time, mRNA expression levels of HIF-1α, a major oxygen homeostasis regulator and endogenous marker of hypoxia widely studied in breast cancer, were inversely correlated with RFS and OS in the same cohort of breast cancer patients.

Moreover, immunohistochemical expression of HIF-1α, revealed a positive correlation with MMP-9 expression in breast cancer cells. This positive correlation was also identified at the mRNA level, after analyzing publicly available RNA sequencing data from a cohort of 971 breast cancer patients. These data provide a possible link between hypoxia, HIF-1α activation and cancer cell invasion, through the up-regulation of MMP-9 in breast cancer.

Conclusions

The results presented in this study provide preliminary evidence for a possible co-expression of HIF-1α and MMP-9 in breast cancer, while underscores the clinical significance of HIF-1a and MMP-9 gene expression in conjunction to RFS and OS. However, immunohistochemistry for HIF-1α and MMP-9 only detects protein expression without determining functional implications in cancer biology, while mRNA expression does not fully reflect protein levels. Therefore, larger scale functional studies are needed in order to clarify the biological significance of different HIF-1α and MMP-9 expression patterns and elucidate whether there is a HIF-1α-induced MMP-9 expression pathway in breast cancer.

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