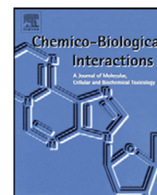
Chemico-Biological Interactions 183 (2010) 455–461

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Expression analysis of maspin in invasive ductal carcinoma of breast and modulation of its expression by curcumin in breast cancer cell lines

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ARTICLE INFO

Article history:
Received 19 July 2009
Received in revised form
19 November 2009
Accepted 19 November 2009
Available online 26 November 2009

Keywords:
Maspin
Invasive ductal carcinoma
Breast cancer
Curcumin
MCF-7
MDA-MB-231

ABSTRACT

In breast cancer, maspin, a serine protease inhibitor, can suppress tumor growth and metastasis *in vivo* and tumor cell motility and invasion *in vitro*. The clinical significance of maspin expression in breast cancer, especially in the sequence of ductal carcinoma in situ (DCIS)–invasive cancer–lymph node metastasis is well known in the Western countries, but its status in the rapidly increasing breast cancers in India remains unknown.

The present study was designed to determine the clinical significance of maspin expression in invasive ductal carcinomas of breast (IDCs) in North Indian population and modulation of its expression by curcumin. Immunohistochemical analysis of maspin showed loss or reduced cytoplasmic expression in 36 of 59 (61%) tumors. Furthermore, breast cancer cells (MCF-7 (wild type p53) and MDA-MB-231 (mutant p53)) were treated with curcumin and the effect on expression of *maspin* gene at transcription and translation levels was analyzed by RT-PCR, immunofluorescence and Western blotting. Maspin expression was also correlated with p53 and Bcl-2 levels. Curcumin inhibited cell growth, induced apoptosis and upregulated maspin gene expression in MCF-7 cells and these findings were further correlated with the upregulation of p53 protein and downregulation of Bcl-2, suggesting maspin mediated apoptosis in MCF-7 cells. To our knowledge this is the first report showing the upregulation of maspin expression by curcumin in breast cancer cells and taken together with the clinical data suggests a potential therapeutic role for curcumin in inducing maspin mediated inhibition of invasion of breast carcinoma cells.

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1. Introduction

Breast cancer is fastly emerging as the most common cancer in women globally. Protease and protease inhibitors are known to play important role in breast tumor invasion and metastasis. Maspin, a 42 kDa protein, belongs to the serine protease inhibitor (clad 5 B serpin) superfamily and is predominantly a cytosolic protein, but is also localized to the nucleus and membrane, and is secreted [1,2]. Maspin, a tumor suppressor protein, is present in high concentration in normal mammary epithelia and accompanying myoepithelial cells, but is downregulated in primary breast cancer cell lines and lost in invasive carcinoma [1,3–5]. In addition to its critical role in mammary gland development, maspin is involved in numerous biological processes including (but not limited to) apoptosis, cell motility, matrix remodeling, angiogenesis, regulation of cell phenotype and redox system [6–8]. Zhang et al. [9] reported that maspin inhibits endothelial cell motility and angiogenesis. Maspin interacts with p53 and may function as an inhibitor of angiogenesis *in vitro* and *in vivo* [10]. Moreover, maspin is under the control of p53 and may therefore not be expressed in tumor cells with abnormal p53 function [10]. It is now established that maspin is epigenetically regulated, and its tissue-specific expression is closely associated with DNA methylation [11,12]. The promoter methylation of *maspin* leads to gene silencing in cancers, such as breast, thyroid, skin, and colon [13–16]. Maspin is implicated in breast cancer, especially in the sequence ductal carcinoma in

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0009-2797/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2009.11.019

situ (DCIS)–invasive cancer–lymph node metastasis in the Western countries [1,4,17].

The established antitumorigenic/antimetastatic properties of maspin in cancer have prompted investigation of its usefulness as a therapeutic agent. Animal studies using targeted delivery of maspin by liposome/DNA and/or adenoviral constructs to tumor and/or tumor vasculature have supported a viable approach in cancer treatment, though limited by safety considerations [18]. Recently, the use of phytochemicals as anti-cancer agents has gained immense importance. Among various naturally occurring phytochemicals, curcumin is capturing the attention of cancer investigators worldwide because of its chemopreventive properties against diverse human malignancies.

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione or diferuloylmethane] has been used in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases [19–23]. Numerous studies have shown that curcumin is a potent inhibitor of proliferation of tumor cells *in vitro* [24,25] and tumor initiation *in vivo* [26–29]. Curcumin is also a potent chemopreventive agent inhibiting tumor promotion in skin, oral, intestinal and colon carcinogenesis [30–32].

Several studies demonstrate that curcumin's anti-inflammatory and anticarcinogenic effects are attributed to its ability to inhibit transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), EGR1, ETS2, signal transducers and activators of transcription (stat), and serine/threonine protein kinases—phosphorylase kinase, protein kinase C, protamine kinase, pp60c-src tyrosine kinase, p44/42 mitogen-activated protein kinase, and c-Jun-NH2-kinase [33–38]. The phase I human trials have shown that curcumin is well-tolerated and lacks toxicity [39,40], but tumors at different sites show varying response to curcumin. In a recent report from our laboratory, we showed curcumin mediated inhibition of cell proliferation and induction of apoptosis via modulation of Wnt/β-catenin signaling in breast cancer [41].

In the present study we investigated the clinical significance of alteration in maspin expression in invasive ductal carcinomas (IDCs) of breast by immunohistochemistry and correlated the findings with clinicopathologic variables (age, histologic grade and tumor size), estrogen receptor α (ERα), BRCA1, erbB2 and p53 expression status. The other aspect of the study was to determine if maspin is a potential molecular target of curcumin in breast cancer. To address this hypothesis, the effect of curcumin on expression of maspin and other maspin-associated proteins was determined in breast cancer cell lines, MCF-7 and MDA-MB-231.

2. Materials and methods

2.1. Chemicals

Curcumin [cat-C7727; ≥94% (curcuminoid content), ≥80% (curcumin)] was purchased from Sigma–Aldrich (Bangalore, India). Anti-human maspin specific antibody (mouse monoclonal, cat-550839) was obtained from BD Pharmingen, San Diego, CA. Anti-p53 (mouse monoclonal, sc-126), anti-Bcl-2 (mouse monoclonal, sc-509), anti-ERα (mouse monoclonal, sc-8005), anti-erb2 (mouse monoclonal, sc-7301) and anti-α-tubulin (mouse monoclonal, sc-5286) antibodies were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Anti-human BRCA1 (OP-92) specific antibody (mouse monoclonal) was obtained from Oncogene Research, Darmstadt, Germany. Biotinylated secondary antibody (LSABTM kit), streptavidin-labeled FITC and fluorescence mounting medium were procured from Dako Cytomation, Glostrup, Denmark. Dulbecco's Modified Eagle Medium (DMEM), Letvobitz-15 (L-15), antibiotics and fetal bovine serum (FBS) were obtained from Sigma–Aldrich, Bangalore, India.

Table 1
Correlations between maspin protein expressions and clinicopathological parameters in patients with IDCs of breast.

Parameter	Total cases (N)	Maspin (Cyto)			Maspin (Nucl)		
		–	+	p	–	+	p
	59	36	23		48	11	
Age (years)							
≤50	29	18	11	NS	22	7	NS
>50	30	18	12		26	4	
Menopausal status							
Pre-	29	19	10	NS	22	7	NS
Post-	30	17	13		26	4	
Tumor grade							
$T_1 + T_2$	35	21	14	NS	27	8	NS
$T_3 + T_4$	24	15	9		21	3	
Lymphatic involvement							
N_0	16	10	6	NS	14	2	NS
N_{1-2}	43	26	17		34	9	
p53							
−ve	30	24	6	.003*	26	4	NS
+ve	29	12	17		22	7	
BRCA1							
−ve	23	16	7	NS	19	4	NS
+ve	36	20	16		29	7	
Her2/neu (Erb2)							
−ve	47	30	17	NS	38	9	NS
+ve	12	6	6		10	2	
ERα							
−ve	37	28	9	.005**	31	6	NS
+ve	22	8	14		17	5	

TNM classification on basis of AJCC classification [42]. *Abbreviations*: Cyto, cytoplasmic; and Nucl, nuclear.
* $p = 0.003$; OR = 5.7 [95%C.I. = 1.776–18.082].
** $p = 0.005$; OR = 5.4 [95%C.I. = 1.727–17.165].

2.2. Clinical specimens

Surgically resected specimens from untreated primary breast carcinomas, and paired normal breast tissues were collected from 58 breast cancer patients enrolled in the Out Patients Department of Surgical Disciplines, Safdarjung Hospital and All India Institute of Medical Sciences (New Delhi, India), after approval of the study by Institutional Human Ethics Committees. Written consent was taken from all the patients enrolled in the study. The age of the patients ranged from 30 to 75 years. All the patients included in this study were invasive ductal carcinoma (IDC) patients and their clinicopathological parameters are summarized in Table 1 (parameters were collected as described by Viale [42]).

2.3. Immunohistochemistry

Paraffin embedded sections (5 μm thickness) of human breast carcinomas and matched normal tissues specimens were collected on gelatin-coated slides. Briefly, the serial sections were deparaffinized in xylene, hydrated and pre-treated in microwave-oven in Tris–Cl buffer (pH 9.0) for antigen retrieval. The sections were then incubated with H_2O_2 (0.3%, v/v) in methanol for 45 min to quench the endogenous peroxidase activity. Non-specific binding was blocked with 1% BSA in PBS for 1 h. Thereafter, slides were incubated with primary antibody overnight at 4 °C. After extensive rinsing with PBS, sections were incubated with biotinylated anti-mouse anti-serum and subsequently with horse-radish peroxidase–streptavidin conjugate (LSAB plusTM kit) (Dako Cytomation, Glostrup, Denmark). Sections were rinsed and color was developed using 3,3′-diaminobenzidine hydrochloride (DAB) as chromogen. Finally, sections were rinsed in distilled

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