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Interleukins-6 and -11 expression in primary breast cancer and subsequent development of bone metastases

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Abstract

Breast cancers frequently metastasize to bone where they often cause extensive tumor-induced osteoclast-mediated osteolysis. Interleukin-6 (IL-6) and IL-11 are two cytokines exhibiting osteolytic properties through their potent stimulation of osteoclast formation. We investigated the expression of IL-6 and IL-11 in 99 invasive primary breast tumors by immunohistochemistry and in situ hybridization, respectively. We examined their potential as predictive factors for further development of bone metastases. 52/90 (57%) of tumor samples showed IL-6 cytoplasmic immunostaining. There was no significant association between IL-6 status and any of the classical prognostic factors. 15/89 (17%) of the tumor samples expressed IL-11 mRNA. A positive IL-11 mRNA status was associated with a low tumor grade ($P = 0.05$). Tumors expressing IL-11 mRNA had a statistically significant ($P = 0.002$) higher rate of bone metastases occurrence (12/15, 80%) than IL-11 negative tumors (27/74, 37%). Such association was not found for IL-6. Our findings demonstrate for the first time IL-11 gene expression in some primary invasive breast tumors and suggest the potential of this cytokine as possible biological predictive factor for the development of bone metastases.

Keywords: Breast cancer; Bone metastases; Interleukin-6; Interleukin-11; Prognostic factor; Osteolysis

1. Introduction

Breast cancer is the most common lethal neoplasm in women. Breast cancer cells (BCC) frequently metastasize to bone and cause tumor-induced osteolysis (TIO). TIO is often characterized by a dramatic stimulation of bone resorption, with little evidence for a normal repair, leading to the considerable morbidity of bone metastases [1]. Although its mechanism is yet to be fully understood, TIO is mainly attributed to the action of the normal bone-resorbing cells, the osteoclasts, whose number and activity are increased near the metastatic foci of BCC, presumably by one or more factor(s) secreted by BCC. One of these factors could be parathyroid hormone-related peptide (PTHrP). However, while experimental support has been obtained for a local role of PTHrP in bone metastases formation, patients with PTHrP-positive primary

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cancers appear less likely to develop such metastases at any time during observation after surgery [2,3].

Interleukin-6 (IL-6) and interleukin-11 (IL-11) are two cytokines acting on various cell types via the same signal transduction pathway involving glycoprotein 130. Besides their immunomodulatory, acute phase reaction and hematopoiesis stimulatory effects [4,5], these cytokines are potent stimulators of osteoclast formation [6,7]. They have been shown to partly mediate bone resorption induced by various osteolytic agents such as PGE_2, IL-1, TNFα and the systemic hormones PTH and calcitriol [8]. We have previously reported that 2 out of 13 BCC lines express and secrete both IL-6 and IL-11 [9,10]. Both cell lines, MDA-MB-231 and Hs578T, have a highly dedifferentiated phenotype including the absence of estrogen receptors and an elevated potential of invasiveness. The injection of MDA-MB-231 BCC into the left ventricle of nude mice induces in a few weeks a dramatic bone destruction [2]. This background led us to hypothesize that IL-6 and -11 expression might be a possible determinant of bone invasion by BCC. We show here that IL-6 and IL-11 mRNA are indeed expressed by some primary human breast carcinomas and we suggest that the expression of IL-11 might predict the occurrence of bone metastases.

2. Patients and methods

2.1. Patient and tumor samples

Paraffin sections of primary breast carcinomas were obtained from women with invasive breast cancer who were operated at Institut Jules Bordet. Tumors were selected as follows: 67 from an adjuvant prospective trial [11] (all patients who developed metastases and with available pathological specimens were included) and the remaining 32 from patients for whom we had paired biopsies (primary tumors and metastatic sites, for future studies). 47 patients had developed bone or bone and visceral metastases as the sites of relapse (20 of them metastasized only in bone) whilst the 52 remaining patients had not developed bone metastases at the time of evaluation i.e. after a median follow-up of 39 months (see Table 1). Ninety and 89 tumor samples out of 99 were evaluated for IL-6 and IL-11 expression, respectively, because of the unavailability of some tumor samples for performing both techniques (77 tumor slides could be analyzed for all parameters). 57% (27/47) and 67% (35/52) of the two groups of patients (metastases in bone or in other sites) were dead by the time of analysis. All patients with bone metastases had lytic or mixed bone involvement. Metastases in bone were assessed by bone scintigraphy, standard X-rays, CT scan when necessary and by ultrasound and/or CT-scan examinations for the other organs.

After fixation in 10% buffered-formalin and paraffin embedding, 5 μm thick tissue sections were stained with hematoxylin and eosine. Tumor histological grading was done using the modified Bloom and Richardson grading system [12]. Steroid receptor status was measured using a dextran-coated charcoal method and Scatchard analysis. Tumors containing hormone receptor levels >10 fmol/mg of protein were considered to be estrogen-receptor (ER) or progesterone-receptor (PR) positive. We also investigated the presence of IL-6 and IL-11 in 10 normal mammary glands obtained from reduction mammoplasty.

2.2. Cell lines and cultures

MDA-MB-231 and MCF-7 BCC lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal calf serum. Cells were harvested by centrifugation. The pellets were embedded in agar and processed in the same way as breast tumor samples. Five-micrometer thick sections were mounted on slides and incubated overnight at 60°C before processing for immunohistochemistry or in situ hybridization.

2.3. Immunohistochemistry for IL-6 detection

Immunohistochemistry for the detection of IL-6 was performed using a mouse monoclonal anti-human IL-6 antibody (Genzyme 1618-01, Cambridge, MA) and a standard streptavidin-biotin-peroxidase technique (Super Sensitive MultiLink-HRP/DAB, Biogenex, San Ramon, CA). Control slides in which IL-6 antibody had been omitted served as negative controls. As positive controls, we confirmed the presence of IL-6 in MDA-MB-231 cells as well as in thyroid tissue and lymph node [9,10,13,14]. Paraffin sections were dewaxed and incubated for 30 min in

Table 1
Patient and tumor characteristics[a]

		Metastases	
	All patients $n = 99$	Bone or bone + other $n = 47$	No bone $n = 52$
Age (years)			
Median	49.5	51	46
Range	29–81	31–81	29–72
Follow-up (months)			
Median	55	50.1	39.1
Range	12.5–117.6	12.5–117.6	13.7–112.9
Menstrual status			
Premenopausal	56	23	33
Postmenopausal	43	24	19
ER status			
Positive (>10)	47	24	23
Negative	36	16	20
Unknown	16	7	9
PR status			
Positive (>10)	51	26	25
Negative	32	14	18
Unknown	16	7	9
T size (pathological)			
≤1 cm	6	1	5
>1 and <2 cm	28	18	10
>2 and <5 cm	39	12	27
>5 cm	5	3	2
Unknown	21	13	8
No. involved nodes			
1–3	33	16	17
4–9	33	13	20
>10	18	9	9
Negative	11	6	5
Unknown	4	3	1
Histology			
Ductal	86	41	45
Lobular	9	5	4
Ductal + lobular	3	1	2
Other	1	-	1
Histological grade			
G1	11	6	5
G2	53	29	24
G3	23	4	19
Unknown	12	8	4

[a] ER, estrogen-receptor; PR, progesterone-receptor.

methanol:H_2O_2 (60:1) to quench endogenous peroxidase activity. Primary antibody was incubated at the dilution 1/50 in 0.3% bovine serum albumin (BSA) in phosphate-buffered saline solution (PBS) for 2 h. Biotinylated multilink second antibody was then added for 30 min followed by streptavidin-HRP for 20 min. All incubations were carried out at room temperature (RT). Slides were washed twice in PBS for 10 min between the different steps. Immunostainings were revealed using 3,3′ diaminobenzidine (DAB) as chromogen. Immunostained slides were counterstained in hematoxylin, dehydrated in serial alcohols and mounted before optical microscope examination.

2.4. In situ hybridization for IL-11 detection

We could not detect the presence of IL-11 by immunohistochemistry because of the unavailability of antibodies against IL-11 protein working with paraffin-embedded samples. We thus developed an in situ hybridization technique. We used oligonucleotide probes for IL-11 mRNA (target region: bases 299-327) [15], supplied doubly labeled with fluorescein isothiocyanate (FITC) (HybriProbe, Biognostik GmbH, Göttingen, Germany) and an amplified immunodetection system for in situ hybridization with non radioactive nucleic acid probes (Super Sensitive ISH detection system, Biogenex, San Ramon, CA). Slides in which IL-11 mRNA probe was omitted served as negative controls. A positive control for assessment of mRNA quality was performed using poly dT-FITC supplied by the manufacturer. We could detect IL-11 mRNA in MDA-MB-231 cells but not in MCF-7 cells, confirming our previous results [9,10]. An additional positive control was obtained by detecting IL-11 mRNA in sarcoid granulomas, as described by other investigators [16].

Slides processed for in situ hybridization were dewaxed and incubated for 30 min in methanol:H_2O_2 (60:1) to quench endogenous peroxidase activity. Sections were then progressively hydrated and digested by proteinase K according to the recommendations of the manufacturer (Biogenex) for 15 min at 37°C. A prehybridization step was done in HybriBuffer ISH (Biognostik GmbH, Göttingen, Germany) by incubating slides in a humidity chamber for 60 min at 37°C. Hybridization was then performed overnight at 37°C by incubating sections with HybriProbe diluted directly in HybriBuffer ISH to a final concentration of 50 pmol/ml. After hybridization, sections were rinsed twice for 5 min in 1× SSC at RT to remove most of the excess probe and then twice for 15 min in 0.1× SSC at 39–41°C. Immunodetection of the FITC label was performed by incubating sections for 30 min with the goat anti-FITC monoclonal antibody at RT, followed by a 30 min incubation with a biotinylated mouse anti-goat $F(ab)_2$ fragment and then by a 20 min incubation with streptavidine-peroxidase. All products needed for detection steps are included in the Super Sensitive ISH detection system. Slides were washed twice for 10 min in PBS between the different steps. Peroxidase was revealed using DAB as chromogen. Slides were then counterstained with hematoxylin, dehydrated and mounted before optical microscope observation.

2.5. Quantitative evaluation of immunohistochemistry and in situ hybridization

The sections were scored after common agreement between two observers (D.L. and C.S.) without knowledge of patient data. The intensity of staining of the breast tumors was graded on a scale of 0 to 3 (0, no positive staining of tumor cells; 1, weak positive staining; 2, moderate positive staining and 3, strong positive staining). The proportion of cells positively stained was graded from 0–4 (0, no positive cells; 1, $<$10%; 2, 11–50%; 3, 51–75% and 4, 76–100%). The two scores were then added to yield a total score (0–7) as described by others [17]. Immunostaining was then considered as negative when the total score was 0, weak when it was 1–3 and strong when it was $\geq$4.

2.6. Statistical analysis

Univariate association between dissemination type, bone or non-bone metastases, and categorical variables was tested using homogeneity chi square tests (with continuity correction in case of binary variables) or chi square tests for a linear trend in case of ordinal variables. Association with a continuous variable was tested using an analysis of variance. When the test for association provided a value of $P < 0.30$, the corresponding variable was selected to be tested in multivariate setting using logistic regression models (a final model was selected using a backward stepwise method and tests on the model coefficients based on the likelihood ratio). The modeled probability was the probability to disseminate in a bone site. All reported probabilities are two-sized and the level for statistical significance was 5% [18,19].

3. Results

3.1. IL-6 and IL-11 expression in breast carcinoma

Patient and tumor characteristics are summarized in Table 1 (left column). Expression of IL-6 was evaluated in 90 of them: 52/90 (57%) showed a positive IL-6 immunostaining. An example of IL-6 staining in an invasive ductal carcinoma is presented in Fig. 1A.

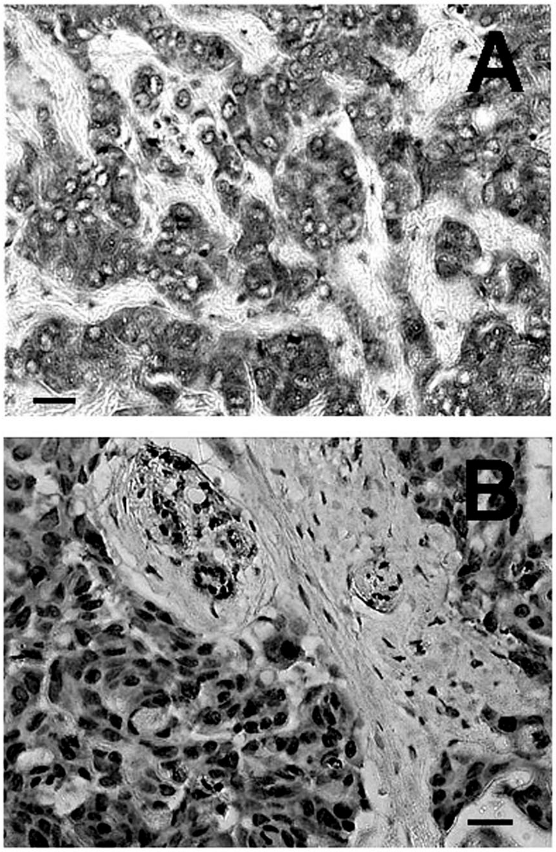

Fig. 1. (A) Immunohistochemical cytoplasmic staining for Il-6 in invasive ductal carcinoma. (B) Cytoplasmic pattern of staining by in situ hybridization for IL-11 mRNA in invasive ductal carcinoma. Bar, 25 μm.

There was no statistically significant association between IL-6 status and any of the classical prognostic factors except for a positive trend in favor of the presence of ductal tumors ($P = 0.07$). We also investigated IL-6 expression in 10 normal mammary specimens: 5/10 (50%) of them were positive for IL-6 immunostaining. In tumor as well as in normal tissues, the labeling was homogeneous and localized to the cytoplasm (Fig. 1A).

IL-11 mRNA expression was evaluated in 89 specimens. 15 out of the 89 cases (17%) showed a positive staining for IL-11 mRNA. An example of IL-11 staining in an invasive ductal carcinoma is presented in Fig. 1B. As for IL-6, the staining was localized to the cytoplasm and was homogeneous. As shown in Table 2, there was a significant association between low tumor grade (grades 1–2) and positive IL-11 status ($P = 0.05$). Ten out of fifteen (66%) IL-11 positive tumors also expressed IL-6. Lastly and contrarily to IL-6, all ten normal mammary tissue specimens were negative for IL-11 transcript staining.

3.2. Association between IL-6 and IL-11 expression, clinical and prognostic factors, and further development of bone metastases

We studied the possible association between IL-6 and IL-11 expression, as well as various prognostic factors, and the type of tumor dissemination (bone sites only or bone together with other sites versus other metastatic sites than bone). There was only a trend in favor of the development of bone metastases

Table 2
Association between IL-11 expression and clinical and pathological parameters

	IL-11 + $n = 15$	IL-11 − $n = 74$	P^a
Age (years)			
Median	51	48	0.09
Range	41–75	29–81	
Menstrual status			
Premenopausal	6	43	0.26
Postmenopausal	9	31	
ER status			
Positive (>10)	8	35	1.0
Negative	5	27	
Unknown	2	12	
PR status			
Positive (>10)	11	36	0.11
Negative	2	26	
Unknown	2	12	
T size (pathological)			
<2 cm	4	25	1.0
>2	5	37	
Unknown	6	12	
No. involved nodes			
≤3	5	31	1.0
>4	6	42	
Unknown	4	1	
Histology			
Ductal	14	65	1.0
Other	1	9	
Histological grade			
G1 + 2	10	45	0.05
G3	0	22	
Unknown	5	7	

[a] Significance calculated with the Fisher test (when indicated, the unknown group is not included).

for IL-6 positive tumors but the value did not reach statistical significance ($P = 0.08$). The same was true for tumors with a high staining score (score > 4). In contrast, univariate analysis revealed a statistically significant association between IL-11 expression and dissemination type. Tumors expressing IL-11 mRNA had a statistically significant ($P = 0.002$) higher rate of bone metastases (12/15, 80%) than IL-11 negative tumors (27/74, 37%). In addition, IL-11 mRNA positive tumors which had metastasized to bone exhibited more often a high staining score (score > 4) than those tumors which had metastasized elsewhere, 4/12 versus 0/3.

Among classical prognostic factors, only tumor grade was significantly associated with further development of bone metastases. Grade 1–2 tumors had a higher rate of bone metastases development (35/64, 55%) than grade 3 tumors (4/23, 17%) ($P = 0.002$). There was no significant correlation between the development of bone metastases and any of the other studied variables, namely age ($P = 0.45$), menopausal status ($P = 0.21$), histology (ductal versus others, $P = 0.85$), number of positive nodes ($P = 0.51$), presence of ER ($P = 0.55$), presence of PR ($P = 0.52$), and tumor size ($P = 0.88$).

Following this univariate analysis, grade, menopausal status, IL-6 and IL-11 were included in a multivariate model (see Section 2). IL-11 mRNA expression and tumor grade remained significant independent predictive markers for further development of bone metastases using the logistic regression model. Grade 3 tumors, whatever the IL-11 mRNA status, metastasized less often to bone sites (OR of 0.18, 95% CI: 0.05–0.70,$P = 0.006$), while patients with IL-11 mRNA positive staining, whatever the grade of the tumor, developed more often bone metastases (OR of 3.81, 95% CI: 1.0–15.7, $P = 0.05$). Finally, the propensity of IL-11 positive tumors to metastasize in bone was not affected by the co-expression of IL-6 ($P = 0.36$).

4. Discussion

We evaluated by immunohistochemistry or in situ hybridization the expression of IL-6 and IL-11 in primary breast cancer specimens. Furthermore, we tested the hypothesis that this expression might predict the development of bone metastases. 57 and 17% of our series of breast tumors expressed IL-6 protein and IL-11 transcript, respectively. Our data on IL-6 agree with a previous report showing that breast tumors can indeed produce this cytokine [17]. In contrast, to the best of our knowledge, our work is the first demonstration of the presence of IL-11 mRNA in some primary breast cancers. It could be worthwhile to investigate the role of IL-11 on breast tumor growth as it has been described for IL-6 [20]. Indeed, the growth of breast cancer cell lines positive for estrogen receptors is usually reduced by IL-6, whereas estrogen-receptor-negative (more aggressive) cell lines are frequently found to be resistant to IL-6. In an in vitro study involving 13 BCC lines, we showed that IL-6 and IL-11 were expressed at both the mRNA and protein levels only in the same two cell lines [9,10]. In the present work, although 66% of IL-11 positive tumors also produced IL-6, there was a substantial proportion of IL-6 positive tumors (69%) in which IL-11 mRNA was undetectable. This suggests that results obtained in breast cancer cell lines account only partially for the complexity of cytokine expression by tumors in vivo.

In contrast with IL-6, none of the ten analyzed normal breast tissues was positive for IL-11 mRNA staining. This suggests that the expression of the IL-11 gene in BCC could constitute a property acquired during the process of carcinogenesis. This deserves further studies examining the as yet unknown mechanisms of IL-11 gene transcription in BCC lines and in breast tumors.

It has been previously shown that patients who will develop bone metastases are more likely to be older, to be post-menopausal with lobular carcinoma, to have little or no involvement of axillary lymph nodes, and low grade tumors [21]. Conflicting data have been published regarding the ER or PR status and the further development of bone metastases [22–25]. In our series, of the classical prognostic factors, only tumor grade (grade 1–2) was an independent marker of the further development of bone metastases. A positive trend was observed between IL-6 expression by breast tumors and the development of bone metastases, but this association was not statistically significant ($P = 0.08$). By contrast, we found a significant correlation ($P = 0.002$) between the presence of

IL-11 in tumors and further development of bone metastases. Moreover, all of the IL-11 positive tumors had a low-grade tumor status, while no association was found between IL-6 status and tumor grade as it was reported by others [26]. These data have obviously to be confirmed, because of the small number of breast tumors positive for IL-11.

What is the functional link between IL-6 and -11 expression by breast cancer cells and their propensity to colonize the skeleton? Bone is a hard-mineralized tissue and its invasion by tumor cells present in the bone marrow implies the action of the normal bone-degrading cells, the osteoclasts [27]. Among osteoclast-activating factors produced by BCC, parathyroid hormone-related peptide (PTHrP) could play a significant role. However, while experimental support has been obtained for a local function of PTHrP in bone metastases formation, patients with PTHrP-positive primary cancers appear less likely to develop such metastases at any time during observation after surgery [2,3]. On the other hand, IL-6 and IL-11 are important factors for osteoclast formation [7]. Our results suggest that, besides their induction of IL-6 and IL-11 production by osteoblasts through the release of PTH/PTHrP and other factors [28], BCC could also directly secrete these cytokines in the bone microenvironment. This would provide them an additional advantage to induce local osteolysis and the release of stimulatory growth factors from the bone matrix.

Besides osteoclast activation, BCC-derived interleukins could also contribute to the development of bone metastases by modulating the function of osteoblasts. IL-11 appears to inhibit bone formation in vitro [29] and to enhance osteoblast-mediated osteoid degradation [30]. We have previously shown that culture medium conditioned by the IL-6 and IL-11-producing MDA-MB-231 BCC was able to stimulate, in absence of osteoclasts, the degradation of collagen synthesized by osteoblasts [31].

Our data are consistent with a recent abstract reporting that increased IL-11 expression by MDA-MB-231 BCC resulted in increased bone metastases in inoculated nude mice [32]. A role of IL-6 and IL-11 in the development of bone metastases is also suggested by our previous work showing that the aspirin metabolite salicylate inhibited the production of these cytokines by MDA-MB-231 and Hs578T BCC [10]. Aspirin was previously shown to inhibit bone deposits and osteolysis in animals injected with various tumor cell types, including BCC [33,34].

IL-6 and IL-11 act on their target cells via a complex signal transduction pathway involving at least one ligand-binding subunit ('receptor') and the common glycoprotein 130 (gp130). Two forms of the IL-6 receptor have been identified: the soluble form (sIL-6R) is generated by shedding of the membrane-bound form. It appears that IL-6 needs the presence of sIL-6R to induce osteoclast formation. A such requirement has not been observed for IL-11. Indeed, a natural soluble form of the IL-11 receptor (IL-11R-alpha) has, to our knowledge, not been described in man [35,36]. sIL-6R production has been found in BCC lines as well as in polymorphonuclear cells from breast cancer patients [20,37]. Further studies should examine the correlation between both IL-6 and sIL-6R expression and bone metastasis in breast cancer patients.

Skeletal colonization by BCC remains associated with a considerable morbidity. However, advances have been made in the development of specific molecules, such as bisphosphonates [38,39], which are able to prevent to some extent the formation of bone metastases. It is thus essential to identify early those patients who are most likely to develop bone metastases. Our results suggest that, besides PTHrP and, maybe, bone sialoprotein [40], IL-6 and, above all, IL-11 expression by BCC might be useful biological predictive factors for the occurrence of bone metastases.

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