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The Relationship Between Bone Metastasis from Human Breast Cancer and Integrin $\alpha_v\beta_3$ Expression

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Abstract. *Background: Osteoclast activation plays an essential role in the development of bone metastasis (BM). Integrin $\alpha_v\beta_3$ mediates the attachment of osteoclasts to bone matrix, and it is overexpressed in bone-residing breast cancer cells. We studied BM from breast cancer in relation to its Integrin $\alpha_v\beta_3$ expression. Materials and Methods: Integrin $\alpha_v\beta_3$ expression in 4 human breast cancer cell lines (MDA-MB-231, MDA-MB-435, MKL-4 and T-47D) was determined by immunohistochemistry, flow cytometry and reverse transcriptase-polymerase chain reaction (RT-PCR). Results: Integrin $\alpha_v\beta_3$ was expressed in MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D, using both immunohistochemistry and flow cytometry. By RT-PCR, positive bands for both α_v and β_3 subunits were identified in MDA-MB-231 and 435, while only the α_v subunit mRNA was present in MKL-4 and T-47D. BM was common in vivo following inoculation with MDA-MB-231 or 435, but not with MKL-4. Conclusion: Integrin $\alpha_v\beta_3$ expression appears to play a key role in the development of BM from breast cancer.*

Bone is the most common metastatic site in breast cancer. Among primary breast cancer patients, 30% develop bone metastasis (BM) as the first event and 70% have BM at autopsy. However, the process of establishment of BM in bone and the bone marrow is not well understood. Integrins are alpha, beta heterodimeric transmembrane proteins. Integrins mediate adhesion to the extracellular matrix and cell-to-cell adhesive interactions (1). We know that Integrin $\alpha_v\beta_3$ mediates various biological events, including the adhesion of osteoclasts to the bone matrix and angiogenesis, among others. For osteoclastic activation in bone matrix to occur, osteoclasts must adhere to bone matrix through Integrin $\alpha_v\beta_3$. Helen *et al.* (2) showed that Integrin $\alpha_v\beta_3$ is overexpressed by bone-residing breast cancer cells and suggested either that subclonal selection of Integrin $\alpha_v\beta_3$ -expression tumor cell populations or up-regulation of Integrin $\alpha_v\beta_3$ in the bone microenvironment must take place for BM to occur. Breast cancer cells adhere to the interstitial space of the bone matrix, but they lack the ability to resorb bone matrix by themselves. So breast cancer cells must be assisted by osteoclasts that can resorb bone matrix (3). If, by expressing Integrin $\alpha_v\beta_3$, cancer cells can adhere to bone matrix, they are likely to interact with osteoblasts and osteoclasts. In this study, we sought to determine whether Integrin $\alpha_v\beta_3$ is an adhesion molecule related to the establishment of BM from human breast cancer.

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Key Words: Bone metastasis, breast cancer, integrins, osteoclasts, osteoclast activation.

Materials and Methods

Animals. Eight-week-old nude rats were purchased and housed at the Laboratory Animal Center of Keio University, Japan, in a pathogen-free environment for the duration of the experiment. The Institutional Review Borad of the animal facility approved the study protocol.

Cell lines. Four different breast cancer cell lines were studied: MDA-MB-231 (ATCC HTB 26), a human breast adenocarcinoma isolated from a pleural effusion; MKL-4, a cotransfectant of the MCF-7 cell line with fgf-4 and lacZ, which micrometastasises to several organs (gift of Dr. Kurebayashi, Kawasaki Medical School, Japan); T-47D (ATCC HTB 133), a human breast ductal carcinoma isolated from a pleural effusion; and MDA-MB-435 (ATCC HTB 129), a human breast ductal carcinoma isolated from a pleural effusion.

Cell culture. Cells were grown in Eagle's minimal essential medium (E-MEM) or Dulbecco's modified Eagle medium (D-MEM) in a gas mixture of 5% CO_2 and 95% air at 37˚C. Passage and medium exchange were performed once a week.

Immunohistochemistry. Cells on the slide were frozen and stained with anti-Integrin $\alpha_v\beta_3$ monoclonal antibody LM609 (1:100) and anti-Integrin $\alpha_2\beta_1$ monoclonal antibody BHA2.1 (1:100). The cells

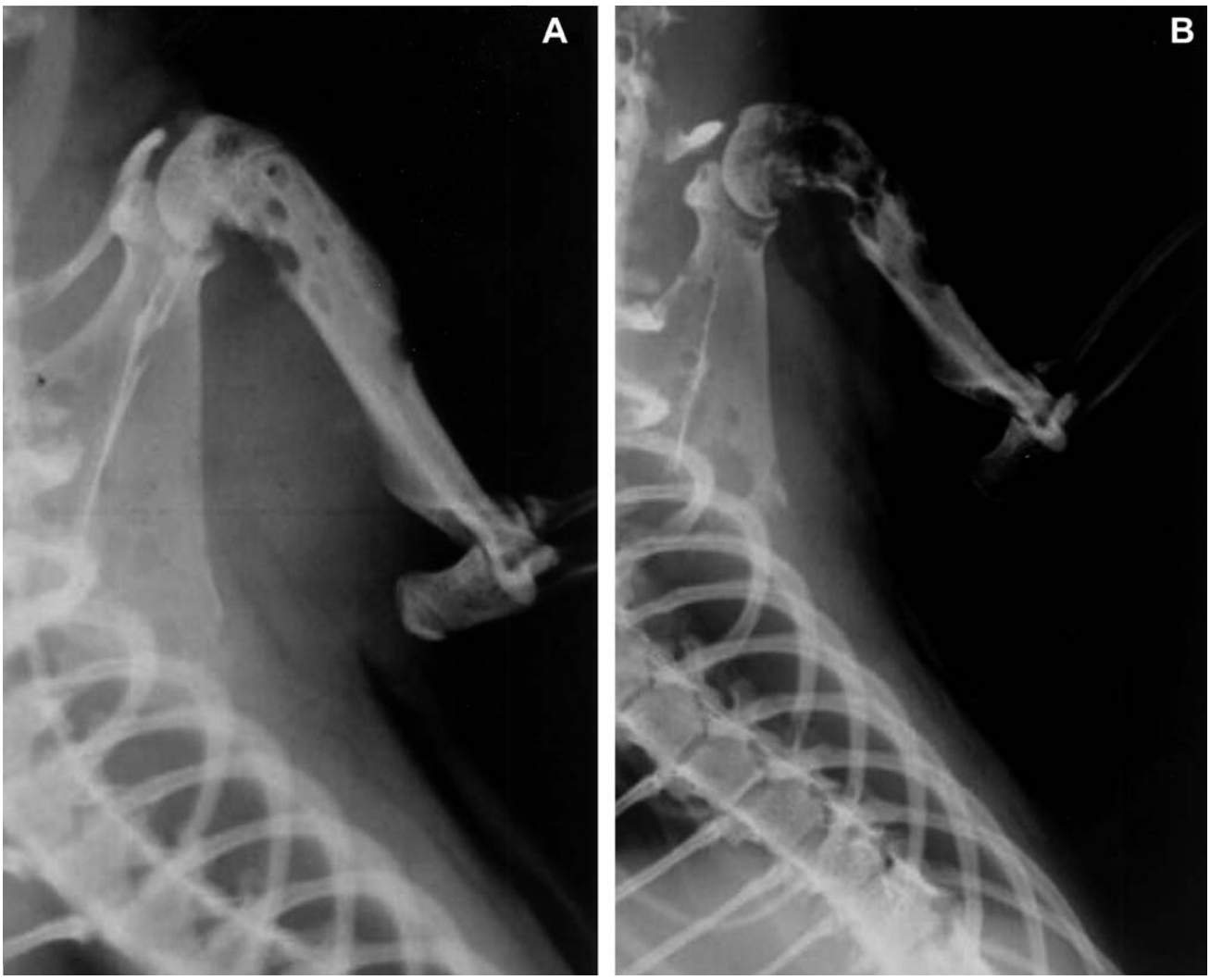

Figure 1. *Roentgenograms of rats with bone metastasis after injection of MDA-MB-231 cancer cells at A : 4 weeks and B: 6 weeks.*

were fixed in acetone for 30 sec, air-dried for 2 min, washed by Tris-buffered saline with calcium (TBS-Ca) for 2 min, blocked with 3% H_2O_2 for 10 min, washed twice with TBS-Ca for 5 min, and then incubated with LM609 or BHA2.1. overnight at 4˚C. The next day, the slides were washed three times with TBS for 5 min, placed in ENVISION+ (Dako, Glostrup, Denmark) that had polymer-immuno complex at room temperature for 30 min, again washed three times with TBS for 5 min, and stained by diaminobenzidine (DAB) for 30 sec.

Flow cytometry. Cells were stained with 5 μg/mL mouse mAb LM609 specific to Integrin $\alpha_v\beta_3$ or mouse mAb BHA2.1 specific to Integrin $\alpha_2\beta_1$ and incubated with anti-mouse-IgG FITC conjugate F(ab')2 Fragment (Sigma, St Louis, MO, USA). Viable cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Population gates were set using cells incubated with normal mouse IgG (4).

Reverse transcriptase –polymerase chain reaction (RT-PCR). RNA was isolated from human breast cancer cells. One microliter of cDNA was used per 25 μL of PCR reaction mixuture. The reaction mixuture contained TaKaRa Z-Taq, 10 X Z-taq buffer, dNTP mixuture, $MgCl_2$ and PCR enhance (TaKaRa, Shiga-ken, Japan). We used the sequence reported in Baljit *et al.* (5) for αv and in Illera *et al.* (6) for β_3. The 5' primer for α_v was 5'-GACTGTGTGGAAGACAATGTCTGTAAACCC, and the 3' primer was 5'-CCAGCTAAGAGTTGAGTTCCAGCC; the size was 305 bp. These primers were extended from human αv cDNA (GenBank accession no. M14648). For β_3, the 5' primer was 5'-CTGGTGTTTACCACTGATGCCAAG, and the 3' primer was 5'-TGTTGAGGCAGGTGGCATTGAAGG and the size was 393 bp. These primers were extended from human β_3 cDNA (GenBank accession no. L28832). PCR amplification for α_v was performed using a three-step process: i) initial extension at 94˚C, for 1 min; ii) annealing at 65˚C, for 1 min; and iii) final extension at 72˚C, for 2

min, and for β_3: i) initial extension at 95˚C, for 10 min; ii) annealing at 60˚C, for 2 min; iii) annealing at 72˚C, for 3 min; and iv) final extension at 72˚C, for 10 min. The amplified products were separated on 1% agarose gels using a 1-kilobase DNA ladder for size comparision.

Surgical procedure and evaluation of BM. The procedure was performed as described in detail elsewhere, with minor modifications (7). Briefly, after anesthetizing 8-week-old nude rats by intraperioneal injection of ketaral 0.7 mL, a small incision was made in the left cervical skin under a dissecting microscope. A polyethylene catheter (PE-10, Becton Dickinson) was inserted into the thoracic aorta from the left common carotid artery. Cells (10^6 or $5x10^6$ suspended in 0.2 mL of Hank's balanced salt solution) were injected slowly into the thoracic aorta. Then the catheter was removed, and the left common carotid artery was ligated.

Whole body radiographs were taken 4 and 6 weeks after injection to identify BM. X-ray confirmed the detection of the lesions (Figure 1).

Results

Immunohistochemical study identified the overexpression of Integrin $\alpha_v\beta_3$ in two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T47D (Figure 2). On flow cytometry, Integrin $\alpha_v\beta_3$ was detected in 82% and 92% of MDA-MB-231 and MDA-MB-435 cells, respectively, and in 26% and 3% of MKL-4 and T-47D cells. α_v and β_3 subunit mRNA was isolated from each human breast cancer cell line by RT-PCR study. Positive bands for both the α_v and β_3 subunits were noted in MDA-MB-231 and 435, while only the α_v subunit was detected in MKL-4 and T-47D (Figure 3).

These three studies were consistent in showing that Integrin $\alpha_v\beta_3$ is expressed in MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D. Further, Integrin $\alpha_2\beta_1$ was overexpressed in all human breast cancer cell lines on both immunohistochemical study and flow cytometry study (Table I).

In vivo, the frequency of developing BM using MDA-MB-231 cells was high [9/11 (81.8%)].We were able to develop BM [8/10 (80.0%)] in rats using MDA-MB-435 cells, as well. Attempts to develop BM using MKL-4 failed [1/9 (11.0%)] (Table II).

Discussion

This study investigated the relationship between bone metastasis from breast cancer and Integrin $\alpha_v\beta_3$. We previously reported that tartrate-resistant acid phosphatase (TRACP) is a useful marker for metastatic bone disease and of the response to treatment in breast cancer, and that both urinary pyridinoline and urinary deoxypyridinoline correlated with the volume of bone metastasis in a rat model (8). Those results showed that tumor cells initially produce micrometastatic foci in the bone marrow, followed by osteoclastic bone resorption, activated by humoral factors that are released from tumor or stromal cells. Recently, Integrin $\alpha_v\beta_3$ has been shown to play an important role in the attachment of osteoclasts to bone matrix (9), and the stimulation of Integrin $\alpha_v\beta_3$ expression by human osteoclasts stimulates adhesion and migration in the bone matrix (10). Currently, Integrin $\alpha_v\beta_3$ antagonists are being developed as inhibitors of bone resorption (11). On the other hand, breast cancers commonly cause osteolytic metastasis according to the type of interaction between breast cancer cells and osteoblasts and osteoclasts (3). Breast cancer cells residing in bone overexpress Integrin $\alpha_v\beta_3$, suggesting either a subclonal selection of Integrin $\alpha_v\beta_3$ -expressing tumor cell populations or up-regulation of Integrin $\alpha_v\beta_3$ in the bone microenvironment. We assumed that, if breast cancer cells overexpressed Integrin $\alpha_v\beta_3$, they are more likely to adhere to bone matrix and interact with osteoblasts and osteoclasts.

We studied the expression of Integrin $\alpha_v\beta_3$ in four human breast cancer cell lines in various ways. Integrin $\alpha_v\beta_3$ was expressed in two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D. This result is consistent with a previous report (12). Thus, it is clear that different human breast cancer cell lines differ in their expression of Integrin $\alpha_v\beta_3$, though all human breast cancer cell lines expressed Integrin $\alpha_2\beta_1$. Integrin $\alpha_2\beta_1$ is a collagen receptor which is commonly expressed in human breast cancer and its loss has been reported to be associated with the invasive phenotype of breast cancer (13). However, in the present study, Integrin $\alpha_2\beta_1$ was not related to BM.

Our attempts to create a model of BM using MDA-MB-231 and 435 cells with Integrin $\alpha_v\beta_3$ were successful. We considered the seed and soil theory consistent with our data (14), *i.e.*, both cells are likely to adhere to bone matrix only when the bone marrow environment had the ligand for Integrin $\alpha_v\beta_3$, osteopontin.

It is reasonable to question the homology between human and rat cDNA for Integrin $\alpha_v\beta_3$. Dolon *et al.* (15) reported that rat cDNA fragments shared 86 to 91% homology with their respective human sequences. So, at least theoretically, using human cDNA in this rat model is justified.

We successfully developed BM by inoculating rats with MDA-MB-231 [9/11 (81.8%)] and MDA-MB-435 cells [8/10 (80.0%)], both of which express Integrin $\alpha_v\beta_3$. On the other hand, we failed to consistently develop BM using MKL-4 cells which lack Integrin $\alpha_v\beta_3$ [1/9 (11.0%)]. This result shows that expression of Integrin $\alpha_v\beta_3$ by breast cancer cells is critical to the formation of BM.

We have shown that the dis-integrin echistatin, arg-gly-asp (RGD)-containing peptide, blocks migration of

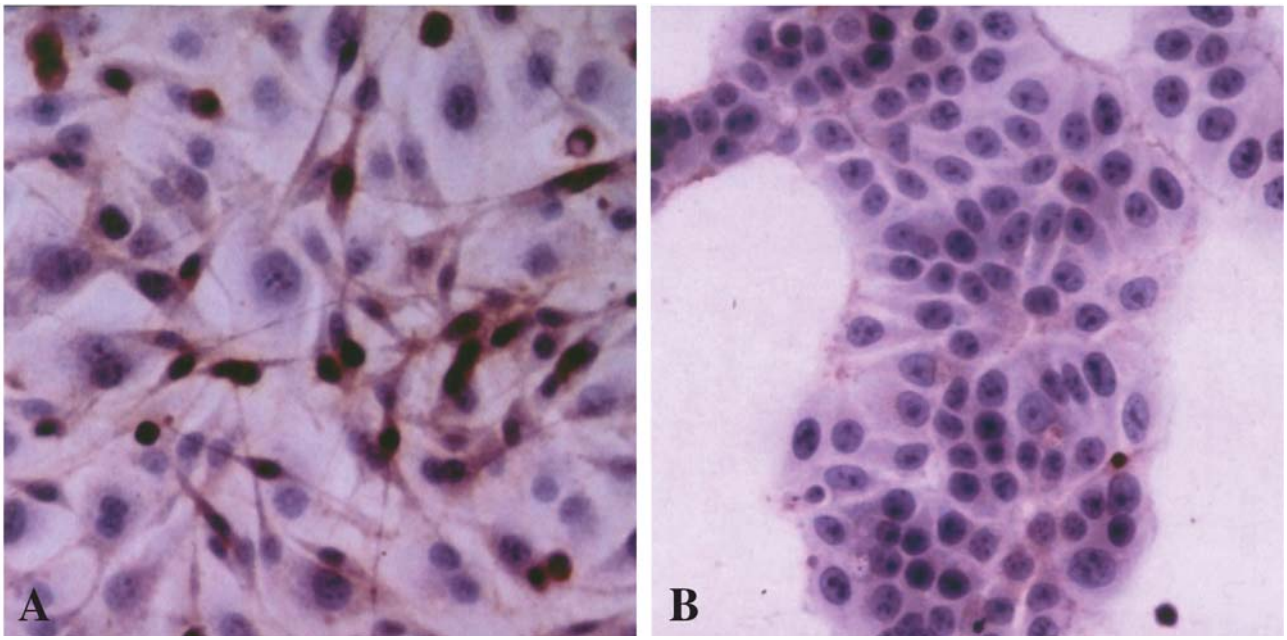

Figure 2. *Overexpression of Integrin $\alpha_v\beta_3$ demonstrated immunohistochemically in A: MDA-MB-231 and B: MKL-4.*

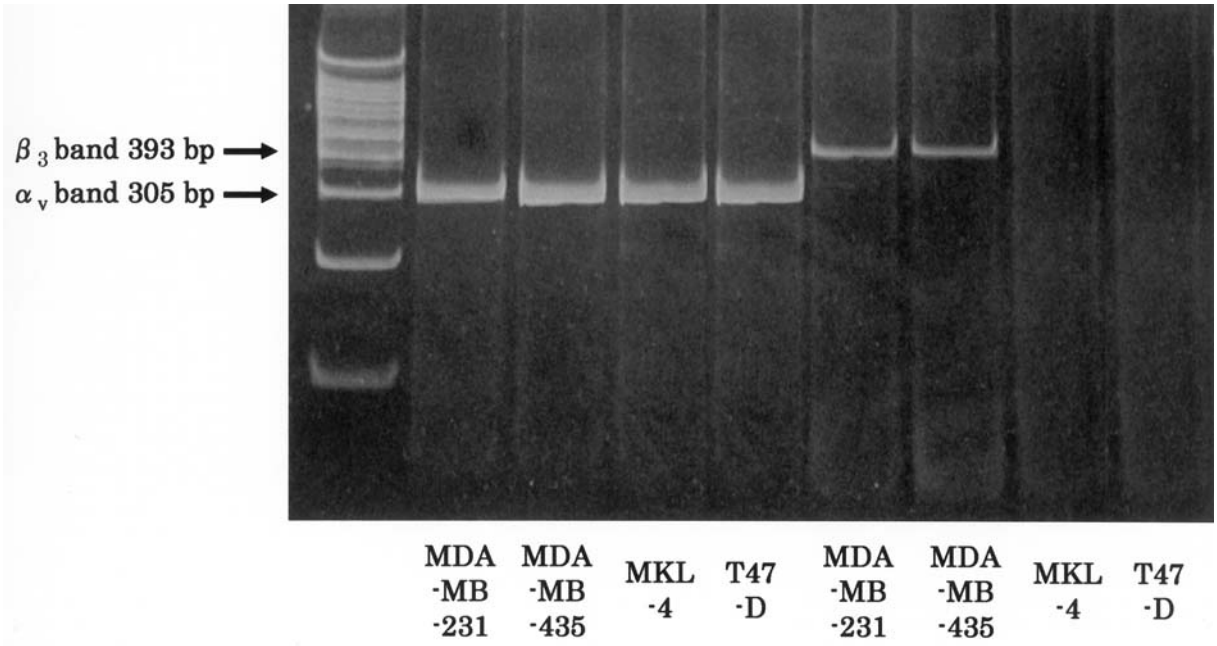

Figure 3. *α_v and β_3 subunit mRNA in human breast cancer cell lines.*

Table I. *Expression of Integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$ in breast cancer cell lines.*

	$\alpha_v\beta_3$	$\alpha_2\beta_1$
MDA-MB-231	+	+
MDA-MB-435	+	+
MKL-4	-	+
T-47D	-	+

Table II. *Frequency of bone metastasis (BM) after injection of breast cancer cell lines.*

Cell lines	Frequency of BM (%)
MDA-MB-231	9/11 (81.8)
MDA-MB-435	8/10 (80.0)
MKL-4	1/9 (11.0)

basement membrane *in vitro*, and the formation of BM using MDA-MB-435 *in vivo* (16). That outcome encouraged us to define Integrin's relationship to BM thoroughly.

The details of the mechanism by which Integrin $\alpha_v\beta_3$ is related to BM are unclear. We do not know whether activation of Integrin $\alpha_v\beta_3$ in human breast cancer cells stimulates adhesion or migration in response to osteopontin *in vivo*, as it does in the osteoclast. The breast cancer cells that express Integrin $\alpha_v\beta_3$ clearly acquire the ability to adhere to the bone matrix. Further study of Integrin $\alpha_v\beta_3$'s role in the development of BM from human breast cancer is required.

Conclusion

1) Integrin $\alpha_v\beta_3$ is expressed in the MDA-MB-231 and MDA-MB-435 human breast cancer cell lines, but not in MKL-4 or T47D.
2) MDA-MB-231 and MDA-MB-435 are more likely to cause bone metastasis in rats than MKL-4.

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