Studies on angiogenic characteristics of bone metastases of human tumours and their modelling *in vivo*

PhD thesis

Tamás Lőrincz, MD

Semmelweis University
School of Ph.D. studies

Project leader:	Miklós Szendrői, MD, PhD, DSc
Consultant:	József Tímár, MD, PhD, DSc
Opponents:	Magdolna Dank, MD, PhD
	József Lövey, MD, PhD
Examination board:	Béla Szende, MD, PhD, DSc
	Imre Antal, MD, PhD
	József Tóvári, PhD

Budapest
2010

Introduction

After the lungs and liver the skeletal system is the third most frequent location for metastasis development of solid malignant tumours, and the metastases of carcinomas are the most frequent malignant tumours of the skeletal system.

At the beginning of our work several experimental data confirmed that beside the destruction of bone matrix, neovascularisation is also a condition for the development of bone metastases. Nevertheless, only limited data were found in the literature about the vascularisation of human bone metastases and their angiogenetic processes. The measurement of angiogenesis is difficult as it is a dynamic process. The majority of the studies focus on the ‘product’ of angiogenesis, the MVD (microvessel density) which corresponds to a snapshot performed at a given time. During our work we have also examined this parameter and the expression of VEGF (vascular endothelial growth factor), an effective pro-angiogenetic factor in bone metastases of human carcinomas.

Furthermore, we have examined HER2/neu expression and gene amplification of bone metastases in human breast cancers. HER2/neu oncoprotein has a role in carcinogenesis; it is a negative prognostic factor associated with visceral metastases and relative resistance to chemotherapies containing cyclophosphamide, methothrexate, 5-fluorouracil or tamoxifen. HER2/neu overexpression is closely associated with increased angiogenesis and it is therapeutic target as well.

Objectives

1. To study the microvessel density in bone metastases of human renal, lung and breast cancers and its comparison with the vessel density in the primary tumour of the same patient
2. To examine the relationship between VEGF expression and microvessel density in bone metastases of human breast cancers
3. To study the possible reasons of vessel density changes in bone metastases of human breast cancers, in the light of the available clinical data

4. To examine the HER2/neu protein expression and gene amplification in bone metastases of human breast cancers, and their comparison with the protein expression and genotype of primary tumours
5. To work out an animal model to study the bone colonisation ability of human tumour cell lines.

Materials and methods

In our studies formalin-fixed, decalcinated, paraffin-embedded samples of bone metastases from a total of 69 cases (10 patients with lung, 11 with renal and 48 with breast cancer) were used. In case of all patients with renal or lung cancer and in 23 patients with breast cancer, samples of the primary tumour were also available, thus we had a possibility to individually compare the primary tumours and their bone metastases.

MVD determination was performed after the labelling of CD34-positive vessels with immunohistochemistry based on 'hot spot' method both in the primary tumours and their bone metastases. VEGF expression was also examined with immunohistochemistry in the primary breast cancers and their bone metastases.

HER2/neu protein expression was determined with immunohistochemistry in the bone metastases of 48 patients with breast cancer. In 23 of 48 cases we had a possibility to also examine HER2/neu expression of the primary tumour giving bone metastasis. Where overexpression or discordance was observed in the HER2/neu status of the individually paired primary tumour and its bone metastasis, gene amplification of HER2/neu was also examined with fluorescent in situ hybridisation.

The bone colonisation of different human tumour cell lines was examined in SCID mice with two methods. In the case of the first model the cell lines were got into the circulation by intracardial injection. In the second model, bone fragments removed during hip prosthesis surgery from human adults were transplanted under the skin of SCID mice, and then human tumour cell lines were injected among the organised bone fragments.

Results

Non-significant change of vessel density was observed when the primary lung, renal and breast cancers and their bone metastases were examined collectively.

Statistically significant correlation between the vessel density of primary tumours and their bone metastases was not found in any histological type.

Vessel density changes observed in the bone metastases compared to the primary tumours were examined separately in the different tumour groups as well. In the case of renal cancers, a significant, 27.59% decrease in MVD was observed in the bone metastases compared to the primary tumours. In the bone metastases the MVD either decreased (in 5 of 11 cases) or did not change (in 6 of 11 cases), and there was no case with increased MVD in the bone metastasis. Not only the primary renal cancers, but their bone metastases also proved to be the most vascularised tumours compared to the lung and breast cancers.

Analysing the lung adenocarcinoma cases, 49.12% MVD increase was observed in the bone metastases compared to the primary tumours. In this tumour type MVD decrease was less frequent (only in 2 of 10 cases), while MVD increase was more frequently observed (in 4 of 10 cases).

In breast cancer cases a non-significant MVD increase was observed in the bone metastases in comparison with the primary tumours. According to the MVD change observed in the paired bone metastases compared to the primary tumours two subgroups were differentiated: in subgroup I the MVD decreased ot did not change in bone metastases, while in subgroup II the MVD increased in bone metastases. It turned out that in subgroup I 6 of 9 patients (67%), while in subgroup II only 2 of 9 patients (23%) received adjuvant therapy. In the bone metastases of patients not receiving therapy the MVD was higher compared to the MVD measured in the paired primary tumours and in bone metastases of treated patients, and these changes were proved to be statistically significant. Moreover, in the bone metastases of patients receiving adjuvant therapy significantly lower MVD was measured than in the primary tumours.

VEGF protein expression was similar in the primary breast cancers and in bone metastases, and no correlation was found between the MVD and VEGF expression.

During the examination of HER2/neu oncoprotein in 48 patients with breast cancer, overexpression and/or gene

amplification indicating HER2/neu targeted therapy was confirmed in 6 cases (12.5%).

In 23 of 48 cases the HER2/neu status of the corresponding primary breast tumours was also determined. By immunohistochemistry 2+ or 3+ expression was observed in 3 of the 23 cases. In all three cases gene amplification was also confirmed with FISH. Apart from these cases, one more gene-amplified primary tumour was found without HER2/neu overexpression. We had 3 of the 23 paired cases where the HER2/neu status of the primary tumour and bone metastasis was discordant from the point of view of HER2/neu overexpression and/or gene amplification indicating anti-HER2 therapy. In most cases, a lower HER2/neu gene copy number was observed in the bone metastases compared to the primary tumours. Moreover, there was no case where gene-amplified bone metastasis was associated with a non-gene amplified primary tumour.

In our experiments aiming at the establishment of *in vivo* models of bone colonisation, we have observed that human melanoma cells injected intracardially into SCID mice colonise the brain, liver and bones. After injection of

transfectants of the WM983B melanoma line, we have noted that the 19L cell line colonised the mouse bones less frequently compared to other transfectants. In the case of intracardial injection of HT168-M1 and HT-199 melanoma cell lines, no gender difference was found regarding bone colonisation.

In our other model applying human bone fragments from adults we have observed that the bone fragments are overgrown by vessel-rich connective tissue after 3 weeks. We have also noted that if 3 weeks after bone fragment transplantation tumour cells are injected among the bone fragments, the melanoma cells colonise the human bone fragments.

6. Conclusions

1. We have determined that the MVD of human breast, renal and lung adenocarcinomas changes in the bone metastases compared to the primary tumours: the mean vessel density in the bone metastases compared to the primary tumours decreased in renal cancers and increased in lung cancers. Our results also show to that in case of

breast cancers the MVD change is influenced by chemo and/or endocrine therapy.

2. We have not found association between the vessel density and VEGF expression of the tumour in the bone metastases of breast cancers suggesting that other angiogenic cytokines may also influence neovascularisation.

3. In our cases breast cancers preserved their HER2/neu-negative status during their progression into the bones. Contrarily, the bone metastases of HER2/neu-positive breast cancers may lose their positivity.

4. Summarizing the above findings, our findings suggest that, if possible, during the metastatic progression of the main human tumour types, pheno- and genotype determination should be repeated from the metastatic tissue, in case this could have therapeutic consequence, since the expression status of proteins used as molecular targets can be different in the metastasis and the primary tumour.

5. By the application of human melanoma cell lines, a bone colonisation model was developed in SCID mice.
6. By the injection of human tumour cells among bone fragments from human adults, implanted into SCID mice, a model can be established which is appropriate for the *in vivo* examination of tumour cell – bone matrix interaction.

Publications

1. **Lőrincz T**, Tóth J, Badalian G, Tímár J, Szendrői M. Her2/neu genotype of breast cancer may change in bone metastasis. Pathol Oncol Res 2006;12: 149-152., **IF: 1.241**

2. **Lőrincz T**, Tóth J, Szendrői M, Tímár J. Microvascular density of breast cancer in bone metastasis: influence of therapy. Anticancer Res 2005; 25: 3075-3081., **IF: 1.604**

3. **Lőrincz T**, Tímár J, Szendrői M. Alteration of microvascular density in bone metastases of adenocarcinomas. Pathol Oncol Res 2004;10: 149-153., **IF: Ø**

4. Döme B, Rásó E, Dobos J, Mészáros L, Varga N, Puskás LG, Fehér LZ, **Lőrincz T**, Ladányi A, Trikha M, Honn KV, Tímár J. Parallel expression of alphaIIbeta3 and alphavbeta3 integrins in human melanoma cells upregulates bFGF expression and promotes their angiogenic phenotype. Int J Cancer 2005; 116: 27-35., **IF: 4.700**