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MARKERS OF METASTATIC BONE PROCESS – TEST OF MULTIPLEX ASSAY

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Bone metastases result in a number of complications in patients including bone pain, pathologic fractures, spinal cord compression, and hypercalcemia, all followed by a decrease in the quality of life (1). Metastatic progression of cancer disease means a worse prognosis and in most cases causes the death of cancer patients. The metastatic spread of cancer to the bones is observed in many malignancies but is mostly related to multiple myeloma, breast, kidney, prostate and lung cancer. The development of metastatic disease is a complex process. It was found that some genes expressed by tumour cells were common for metastases at all sites, e.g. osteopontin, whereas others were selectively expressed if the cell lines had a predilection to growth in a given tissue. The vicious cycle related to bone metastasis develops when factors secreted by or expressed in tumour cells (eg. Parathyroid hormone- related peptide) activate osteoblasts and osteoclasts in the bone microenviroment to produce cytokines (eg. Receptor activator for nuclear factor κB ligand – RANKL); bone remodeling and osteolysis causes the release of growth factors (eg. TGFβ and IGFI), which then stimulate tumour cell growth, motility, and a release of parathyroid hormone related peptide (2, 3).

In the past, there were no therapeutic possibilities for bone metastatic disease, nowadays there is not only palliative therapy but also a curative treatment leading to a stabilized status that acts as a prevention of further development of bone metastasis. Imaging methods, as a conventional measurement of skeletal health and treatment response in metastatic bone lesions, are imprecise and can only detect changes after the damage has occured. Simple tools are required for rapid and sensitive detection of changes in bones during cancer. The science and clinical utility of biochemical markers of bone metabolism are still evolving; therefore, they are not yet an established surrogate measurement for clinical efficacy (4, 5). The rapidly developing multiplex analytic technology opens the door to the multimarker blood monitoring of multifactorial cancer processes. One such tool can be bead-based Multi-analyte profilingtechnology (xMAP) (6).

Presented study is focused on testing the commercially available multiplex Human Bone Panel for the measurement of the marker serum levels by xMAP technology for a detection of tumour induced bone disease (bone metastases) and on comparison to serum bone markers nowadays routinely used in the monitoring of several bone diseases.

METHODS

Patient cohorts

We studied 24 cancer patients with metastatic disease. They were divided into 2 groups: group 1 – tumour disease with occurrence of bone metastases (13 patients) and group 2 tumour disease with no bone metastases (11 patients). A control group of 20 healthy blood donors, referred to as group 0, was also studied. Study has been approved by the ethic committee of the Faculty hospital. The peripheral blood was drawn using VACUETTE® Z. Serum Sep tubes (Greiner Bio-One, Austria) and allowed to clot. Sera were separated by centrifugation and all specimens were immediately aliquoted and frozen. Samples were stored at −80 °C. Before a multiplex analysis the aliquots were centrifuged for 5 min at 10,000g to remove any clots or particles.

Analysis

Serum levels of osteoprotegerin (OPG), osteopontin (OPN), osteocalcin (OC), parathormon (PTH) and leptin were measured by multiplex xMAP technology (Luminex 100 instrument) with use of Human Bone Panel A (Millipore Linco corp., USA) in the cancer and healthy groups. The multiplex analysis was run in duplicates. The following routinely used serum bone markers were assessed in the cancer groups: N-terminal propeptide of type I procollagen (PINP), C-terminal telopeptid of type I collagen (ICTP) and N-propeptide of type III procollagen (PIIINP) (all RIA, Orion Diagnostica, Finland), bone-specific alkaline phosphatase (ostase, known as well as bALP) (automated CLI, DxI UniCell 800, Immunotech – Beckman Coulter comp., USA), C-terminal cross-linking telopeptide of type I collagen (CTx) (ECLIA, Elecsys, Roche, USA) and 25-hydroxyvitamin D (vit.D) (RIA, IDS, UK). Osteocalcin levels were also measured for comparison using both routine immunoassay (RIA, CisBio International, France).and multiplex method.

Results Interpretation

Descriptive statistics were calculated for all markers. The Wilcoxon test was used to compare marker levels between groups. Significance was set for P values lower than 0.05, P values between 0.1 and 0.05 were considered as border significant. For the purposes of the scoring system, the normal values for multiplex markers were set at 95percentil of the healthy group. For non-multiplex markers used cut off values (see in Table 3) were derived from cut offs valid in the routine practise of our laboratory. Each value above normal levels was given a score of 1 point. Two scoring systems for better discrimination of groups 1 and 2 were created by an empirical choice of analytes. Scoring system I – points for osteoprotegerin, osteopontin, PIIINP, ICTP and ostase were counted; in scoring system II – points for osteoprotegerin, osteopontin, PIIINP, PINP and ostase were counted. Because the multiplex is not a routine method, mean coefficients of variation – CV% (relative standard deviation as a percentage) were calculated for all multiplexed analytes. Results falling under the calibration curve ranges were stated as the value of the lowest calibration point. For a comparison of osteocalcin analytical methods Passing Bablock analysis (including Cusum test for linearity) were performed, and Spearman's coefficients of rank order correlation between methods was calculated.

RESULTS

All results for multiplexed markers are shown in table 1 and markers measured by nonmultiplexed methods in table 2. In comparison to the control group, significantly higher levels of osteoprotegerin and significantly lower levels of osteocalcin in both cancer groups were found. No significant differences were found between cancer groups 1 and 2 for any of the multiplexed markers. As for the non-multiplexed markers, group 1 displayed significantly higher levels of PIIINP and higher levels (with border significance) of ostase compared to group 2. Considering the nonmultiplexed markers significantly higher levels of PIIINP and ostase in group 1 compare to group 2 were observed.

Tab. 1 Medians and kvantils for all groups and differences between groups for multiplexed markers

Group			OPG (pg/mL)	OPN (pg/mL)	OC (pg/mL)	PTH (pg/mL)	Leptin (pg/mL)
0	median		262	679	7 838	21	4 552
	kvantil	5%	113	244	4 080	11	244
		95%	639	32 596	13 304	46	17 645
1	median		631	1 978	5 329	22	5 254
	kvantil	5%	218	244	1 017	10	369
		95%	2 417	128 232	22 821	64	22 596
2	median		504	1 742	5 195	24	2 780
	kvantil	5%	268	260	1 723	10	274
		95%	954	6 918	15 594	85	7 426
Group difference Wilcoxon test							
0x1			P<0.0001	P>0.1	P<0.06	P>0.1	P>0.1
0x2			P<0.002	P>0.1	P<0.02	P>0.1	P>0.1
1x2			P>0.1	P>0.1	P>0.1	P>0.1	P>0.1

Tab. 2 Medians and kvantils for cancer groups and differences between groups for non-multiplexed markers

Group			PIIINP (ug/L)	ICTP (ng/mL)	VitD (nmol/l)	PINP (ug/L)	Ostase (ug/L)	CTx (ng/ml)
1	median		8.5	11.0	41.7	49.0	18.4	0.460
	kvantil	5%	3.5	3.9	17.5	35.1	5.3	0.145
		95%	73.1	75.0	74.8	250.0	121.0	1.033
2	median		4.8	8.2	42.0	47.0	8.9	0.420
	kvantil	5%	2.2	4.6	17.1	30.3	5.4	0.163
		95%	15.4	21.4	147.9	77.7	25.1	0.757
Group difference Wilcoxon test								
1x2			P<0.05	P>0.1	P>0.1	P>0.1	P<0.1	P>0.1

Interestingly, 3 of 4 patients with multiple bone metastases had values above the set normal value both for osteoprotegerin and osteopontin in comparison to all other cancer patients, where only one of these markers was positive.

Osteocalcin serum levels obtained by multiplex measurement correlate with routine immunoassay – Spearman's coefficient: R = 0.710 with P value = 0.001. The regression equation obtained by Passing Bablock method for osteocalcin methods: Y = 0.1153

(95% CI: −1.1537 to 0.9909) + 0.2977 (95% CI: 0.2268 to 0.3962) X with no significant deviation from linearity.

Intraassay mean coefficients of variation (CV%) for doublets in multiplex analysis were under 7% for all multiplexed markers except for osteopontin. A worse accuracy was observed for osteopontin: mean CV% 16.9% but with a median of 8.2%, caused by the number of samples with CV% over 15%. No samples with CV% over 15% were observed for leptin. For OPG, one sample, and for OC, two samples, were found to have a CV% of over 15%.

No samples were observed outside calibration ranges for osteocalcin and osteoprotegerin with recommended serum dilution. Three samples were under the calibration curve for leptin and PTH and nine samples for osteopontin in multiplex analysis.

Tab. 3 Cut off values for markers and percents of samples out of set reference ranges in group 1 and 2

Score system	OPG (pg/mL)	OPN (pg/mL)	OC (pg/mL)		PTH (pg/mL)	Leptin (pg/mL)	PIIINP (ug/L)	ICTP (ng/mL)	VitD (nmol/l)	PINP (ug/L)	Ostase (ug/L)	CTx (ng/ml)
Cut off value	>639	>32596	>13304	<4080	>46	>17645	>6.4	>6.0	<23	>22	>100	>0.6
Group 1	46% 6/13	23% 3/13	23% 3/13	38% 3/13	8% 1/13	8% 1/13	54% 7/13	69% 9/13	31% 4/13	38% 5/13	31% 4/13	38% 5/13
Group 2	36% 4/11	0% 0/11	9% 1/11	36% 4/11	18% 2/11	0% 0/11	27% 3/11	64% 7/11	18% 2/11	9% 1/11	0% 0/11	27% 3/11

DISCUSSION AND CONCLUSION

Our study is focused on testing the commercially available multiplex Human Bone Panel for the measurement of the serum levels of OPG, osteopontin OPN, osteocalcin OC, parathormon and leptin by xMAP technology. Our aim was to study the possibility of the detection of tumour induced bone disease using serum tests, to setup the reference serum levels for parameters included in the multiplex panel, and to compare serum bone markers nowadays routinely used in the monitoring of several bone diseases.

In accordance with sources of literature, the most promising of the multiplex markers in our study turned out to be osteoprotegerin, differing significantly in comparison to the healthy group but not between cancer groups. OPG is a soluble decoy receptor for RANKL and so prevents RANKL binding to RANK and the subsequent activation of osteoclast activity. OPG also inhibits apoptosis of tumour cells by inhibiting TRAIL (TNF-related apoptosis-inducing ligand) and resulting in improved survival (7). Furthermore OPG has been found to stimulate angiogenesis, while RANKL is an angiogenic inhibitor (8). In comparison to healthy controls, increased serum OPG levels were observed in prostate cancer, bladder carcinoma, colorectal and pancreatic cancer and were reported to be associated with several other organ systems and pathologies e.g. endometriosis,

periodontal disease, thyroid disease and coronary heart disease (7, 9–10). Serum OPG levels were found to be higher in patients with metastatic bone disease in prostate cancer compared with patients with organ confined disease. Increases in serum OPG may indicate disease progression/relapse in prostate cancer (7, 11–12). In contrast, when analysing sites of metastasis, Lipton et al. only observed a significant elevation of serum OPG levels in patients with liver and soft tissue metastasis and not in patients with bone or lung metastasis (9). On the contrary, a decrease in serum levels of OPG compared to controls was reported in patients with multiple myeloma and in sarcoma patients (7, 9, 13). Some interesting preanalytic information is that OPG levels are higher in serum than in plasma and that the levels are higher in females than in males according to Lipton et al. (9). The age dependence of OPG levels is controversial – age dependence was reported e.g. by Jung et al. and Narita et al. (10, 12) and age independence by e.g. Lipton et al. (9). Unfortunally, cohorts in our study were too small to add further observations to these tasks. Lower levels of osteocalcin in cancer groups, compared to healthy controls, were found in our study, but no difference was found between the bone metastatic group and nonbone metastatic group. Osteocalcin is a small γ carboxyglutamicacid-rich peptide, one of the major noncollagenous proteins of the bone matrix exclusively synthesized by osteoblasts. Serum levels were reported to correlate well with osteoblast activity. In advanced untreated metastatic bone disease, serum osteocalcin levels can be low in the presence of high serum BAP levels. The reasons for this metabolic uncoupling between two bone-formation markers are unclear; however, possibilities include the proteolytic cleavage of osteocalcin, changes in gene expression and disturbed osteoid maturation in the presence of active tumor osteopathy (14–15). On the contrary OC levels were found to be significantly higher in breast cancer patients with bone metastasis compared to non-metastatic or soft tissue metastasis (16). In our opinion multiplex measurement in conjunction with multimarker data handling could, in the future, improve discrimination of bone and non-bone metastatic disease. For purposes of illustration, two scoring systems were created. For scoring system I: a score of 3 or higher was positive for 46% patients in group 1 (6/13) in comparison to 9% in group 2 (1/11); for a score of 2 or higher 61.5% patients (8/13) were positive in group 1 in comparison to 36% (4/11) in group 2. For scoring system II: a score of 3 or higher positive 38% of patients in group 1 (5/13) in comparison to 0% in group 2 (0/11); for a score of 2 or higher positive 54% of patients in group 1 (7/13) contrary to 9% (1/11) in group 2. We observed that patients with multiple bone metastases have values above set normal value for both osteoprotegerin and osteopontin. This fact could help in the discrimination of such patients according to serum tests. Inside the bone osteopontin is produced by both osteoblasts and osteoclasts and has several presumedfunctions: the attachment of osteogenic cells to the bone matrix, control of mineralization, coupling of bone formation and resorption. Elevated OPN levels were described in patients with epithelial ovarian, breast, lung and prostate cancer and were found to be associated with a shorter survival and larger numbers of metastatic sites in breast cancer (17–19). In our study, OPN levels were higher in cancer groups with a very high 95% kvantil in the bone matestatic group, but the differences did not reach statistical significance. Leptin plays a role, not only in the regulation of body weight and energy balance, but also in vascular remodeling, regulating

neoangiogenesis by itself and in conjunction with VEGF and fibroblast growth factor, acts as a mitogen transforming factor and supresses apoptosis. No significant differences among groups were observed in our study and reported studies on serum leptin levels in cancer have produced ambiguous results, which may be caused by pulsatile blood levels (a frequency of about 1 pulse per 45 min.) and diurnal pattern. However, the serum leptin levels were reported to be unaltered or significantly decreased in colorectal cancer and advanced non-small-cell lung cancer in contrast to unaltered or significantly increased leptin levels in breast and prostate cancer. For prostate cancer, higher leptin levels were associated with more advanvanced tumors, characterized by a larger size and higher grade. These facts suggest a possible role played by leptin in metastatic site differences among cancer types, which is why it was included in our multiplex study (20–21).

PTH, the last member of the multiplex panel, is thought to promote the growth and invasiveness of cancer in bone thanks to the increased expression of the PTH receptor in cancer metastases (22), but did not differ between groups in our study.

With regards to the nonmultiplexed markers, significantly higher levels of PIIINP and ostase were observed in bone meatastatic group in comparison to the non-bone metastatic group. Only these two markers discriminate the bone and non-bone metastatic group. No significant differences between bone metastatic and non-bone metastatic patients in any of bone resorption marker (CTx and ICTP) levels were found. Serum levels of ostase were reported to correlate closely with osteoblast differentiation and activity. In most cases of advanced metastatic bone disease ostase levels in serum are elevated, reflecting either a strong osteoblastic component or, in lytic lesions, active repair (14).

According to Demers et al. the best markers for assessing the presence of progression of skeletal metastases appear to be the collagen breakdown products of type I bone collagen (23). In our study we have shown that interest should be focused as well on the propeptide of type III procollagen, which is considered to be a marker of connective tissue metabolism (15) and a marker of liver fibrosis. In our study we have observed elevation of the P3NP in bone-metastatic group in comparison to non-bone metastatic group and OPG in bone metastatic group in comparison to healthy group. Because Lipton et al. observed elevation of serum OPG levels only in patients with liver and soft tissue metastasis and not related to bone disease (9), observations could be influenced by the liver metastatic status of the patients. That is why, in future studies, liver metastases should be precisely considered in larger cohort studies of bone metastatic disease.

There is no chance to make a diagnostic decision considering the bone metastatic disease from only one marker. The bone metastatic development and spread is a consequence of broken balance among cytokines and other proteins regulating osteoblastic and osteoclastic activity that is why the absolute level of one marker is not so important as a ratios among regulation proteins. The only way how to monitor this net regulation is multiplex analysis which enables to measure all markers under the same conditions. This “simulation” of pathofysiological process cannot be sufficiently performed by measurements of markers one by one but only by a multiplex solution. Bone metastases are classified as osteolytic, osteoblastic or mixed. In many cases osteolytic and osteoblastic processes are involved and in fact it is a process wherein both biological situations coexists (1). High bone turnover

with excess bone resorption is therefore an archetypal feature of most, if not all, bone metastasis (14). That is why we do not consider necessary to discriminate cases into osteolytic and osteoblastic cohorts and we believe that with multiplex and multiparameter system is possible in future to develop universal diagnostic and prognostic system.

Studied cohort of patients is quite small and doubtless investigation on larger cohort is necessary but it seems that would be very useful for oncology to incorporate other bone markers e.g. ostase into the multiplex panel. According to literature we conclude that further incorporating of at least RANKL and TGFbeta (3) in future studies is necessary for complex point of view. Doubtless diurnal regime and biologic variability of novel bone markers have to be studied. Furthermore at earlier stages of the disease process changes in skeletal morphology or radionuclide uptake might be discrete, nonspecific or absent and so unidentifiable by imaging methods. We admit the possibility that in group 2 there could be enrolled patients with bone metastasis in development underdiagnosed by imaging methods. For next studies not only the results of imaging methods at the time of blood sampling but as well results of imaging methods couple of months after the blood sampling should be considered. In perspectives multiplex panel will be studied in conjunction with RANKL, TGFbeta and routine bone markers on a larger cohort of patients. We supposed that large cohort study would enable to set up a multiparameter result interpretation system and to choose the best marker combination for multiplex measurement. Such novel complex clinical laboratory tool could be used for early detection of bone metastatic disease, for treatment efficacy monitoring, for predicting of risk of bone metastases development for better treatment tailoring and even for control of up-to-date possibilities in prevention of metastatic disease occurrence.

The treatment monitoring and tailoring is one of the most desired future function of markers in bone metastatic disease management. For example Lester et al. have evaluated a bone marker directed schedule of treatment with zolendronic acid based on levels of the bone resorption marker, urinary N-telopeptide of type I collagen. Their experience suggests that a tailored approach to bisphosphonates therapy may be a more cost-effective approach than the currently licensed and recommended fixed schedule of intravenous treatment (24). The treatment monitoring would enable to improve the cost-effectivity, to reduce the doses by individual tailoring and hopefully to decrease the severity of adverse therapy effects. Nowadays the necessity of proper patient choice for treatment by novel targeted antibodies drugs is coming up. The trouble is that the bone markers are mainly studies separately, e.g. NTx levels in BISMARK study (4) or in a group of 2 to 3 markers. The comparability of results among studies is very problematic. Even the conditions for patient enrolment into a study widely vary. The assessment of a panel of bone markers should be a part of clinical trials of newly introduced drugs, which have a multicentric manner and precisely defined patient cohorts.

Multiplex bone metastasis detection by serum test in future would have advantages of easiness of venous blood sampling, no radiation exposure of patients in comparison to imaging methods, the possibility of regularly monitoring of the therapy efficacy, the monitoring of whole body bone remodelling not only the imaged area, better cost-benefit ratio than single analytical methods and hopefully it can help with creating of

multiparametric scoring system with sufficient sensitivity and specificity for clinical practise.

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Conflict of Interest Statement

The authors have no personal, professional or financial involvement with the matters at issue in the investigation that might create an appearance of bias or actual bias. Authors have full control of all primary data and they agree to allow the journal to review their data if requested.

SUMMARY

A multiplex panel for the measurement of serum levels, including osteoprotegerin, osteopontin, osteocalcin, parathormon and leptin, was tested in a group of cancer patients with metastatic disease, and also in healthy controls. Cancer cases were divided into one group with an occurrence of bone metastases and into one group without bone metastases. Serum levels of markers of biologic activity were measured using Human Bone Panel for multiplex xMAP technology. Additionaly, routine serum bone markers (PINP, PIIINP, ostase, ICTP and 25-hydroxyvitamin D) were assessed with routine immunoassays in cancer groups. In comparison to controls, both cancer groups were observed to have higher levels of osteoprotegerin and lower levels of osteocalcin. The bone metastatic group had higher levels of PIIINP and ostase than the non-bonemetastatic group. 3 of 4 patients with multiple bone metastases had values above the set normal value for both osteoprotegerin and osteopontin in comparison to all other cancer patients. Multiplex bone metastasis detection by serum test in future could help with creating of multiparametric scoring system with sufficient sensitivity and specificity for clinical practise. Multiplex is a powerful tool for multiparametric studies, but we are still in the era of looking for the right marker combinations for cancer diagnostics and monitoring. The panel will be tested on a larger cancer cohort and in patients with non-cancerous diseases.

Markery metastatické kostní choroby – Multiplexová analýza

SOUHRN

U pacientů s nádorovým onemocněním a skupiny zdravých kontrol byly stanoveny multiplexovou metodou vybrané sérové markery biologické aktivity: osteoprotegerin,

osteopontin, osteokalcin, parathormon a leptin a posouzena možnost jejich využití v diagnostice kostní metastatické choroby. Pacienti s nádorovým onemocněním byli rozděleni do skupin s a bez kostních metastáz. Sérové hladiny markerů byly naměřeny soupravou Human bone panel pro multiplexovou analýzu xMAP. K tomu byly v rutinním provozu stanoveny markery kostního obratu: P1NP, P3NP, ostáza (kostní isoenzym alkalické fosfatázy), ICTP a 25-OH hydroxyvitamin D. Výsledky: Obě skupiny pacientů s nádorovým onemocněním měly signifikantně zvýšené hladiny osteoprotegerinu a snížené hladiny osteokalcinu. Skupina s kostní metastatickou chorobou měla vysoké hladiny P3NP a ostázy ve srovnání se skupinou bez kostních metastáz. 3 pacienti ze 4 s mnohočetným kostním postižením měli jak OPN tak OPG mimo rozmezí definované u zdravé skupiny. Multiplexové stanovení markerů kostního postižení by mohlo v budoucnu přispět k vytvoření multiparametrického skórovacího schematu, s dostatečnou sensitivitou i specificitou, použitelného pro běžnou klinickou praxi. Multiplexová analýza je silný nástroj a jsme ve stadiu hledání těch správných kombinací markerů pro využití v diagnostice i monitoringu pacientů s nádorovým onemocněním. Tento soubor markerů bude dále testován na větší skupině pacientů s nádorovým onemocněním.

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