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Activation of the endothelin axis in osteoblasts: genomic analysis and identification of biomarkers of osteoblastic metastasis

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Osteoblastic metastases are common in advanced prostate cancer and some cases of breast cancer. It has been shown that uncontrolled bone formation at the site of metastasis is caused by a number of growth factors, in particular endothelin-1 (ET-1). It has been proposed that secretion of ET-1 by prostate cancer cells is a major factor in formation of osteoblastic metastases. A highly specific antagonist of the ETa receptor, ABT-627, has been developed by Abbott for treatment of metastatic prostate cancer.
To gain better understanding of the mechanism of ET-1-induced osteoblast proliferation and to identify biomarkers of osteoblastic metastasis, we studied the effects of ET-1 (±ABT-627) on primary human osteoblasts and mouse osteoblastic cells by measuring the phosphorylation of several pro-growth kinases and by determining transcriptional activation on a genome-wide scale.
We observed rapid phosphorylation of p38, ERK1/2, and JUNK kinases after the ET-1 treatment. These effects were completely blocked by ABT-627. To determine the pathways involved in the ET-1-induced osteoblast proliferation, we used microarray profiling combined with systematic pathway analysis. We observed coordinated induction of genes associated with invasion/metastasis, survival, and osteoblast and osteoclast maturation. Nearly all the genes induced by ET-1 were blocked by ABT-627, implying that all the effects of ET-1 are mediated by the ETa receptor.
The ET-1 signature in osteoblasts contained several genes coding for secreted proteins previously implicated in invasion and metastasis. Their secretion was confirmed by ELISA. These proteins are currently being explored as biomarkers for osteoblastic metastasis and efficacy biomarkers for ABT-627.
Our studies support and refine the existing model of cancer cell/osteoblast interactions in osteoblastic metastases. They also yielded several candidate biomarkers that can potentially be used to monitor the progression of osteoblastic metastases and the efficacy of antimetastatic therapies.

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The Receptor Activator of Nuclear Factor–kB Ligand (RANKL)/Osteoprotegerin (OPG) axis is severely disrupted in patients with solid tumours and osseous metastasis

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Background: The pathophysiology of bone metastasis has not been fully clarified in several solid tumors. The receptor activator of nuclear factor-kB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system has a major role in osteoclastogenesis. Recently, special emphasis has been given in determining the possible mechanisms by which this system affects the process of bone remodeling in cancer. We investigated the role of these molecules along with novel markers of bone turnover in patients with solid tumors metastatic to bone in relation to the type of malignancy and the neoplastic burden to the skeleton.
Material and methods: Blood specimens from 40 patients with breast, lung and prostate cancer metastatic to the skeleton were collected at the time of diagnosis. Levels of soluble RANKL (sRANKL), OPG, sRANKL/OPG ratio, 5b isoenzyme of tartrate resistant acid phosphatase (TRACP-5b), C-terminal cross-linked telopeptide of type I collagen (CTX), C-terminal propeptide of procollagen type I (PICP), osteopontin (OPN), bone alkaline phosphatase (bALP) and osteocalcin (OC) were assessed using enzyme-linked immunosorbent assay (ELISA) and were compared to those of an age and sex-matched healthy control group.
Results: Patients with breast or lung cancer and bone metastasis had elevated serum levels of osteoclastic activity markers, such as sRANKL ($p = 0.004$), TRACP-5b ($p = 0.022$) and OPN ($p < 0.0001$), compared to controls. Furthermore, sRANKL correlated with the extend of metastatic bone burden ($p = 0.035$ in patients with more than ten sites of osseous metastasis). Levels of osteoblastic activity markers were also significantly elevated compared to controls, including OPG ($p < 0.0001$), bALP($p \leqslant 0.0001$) and OC ($p \leqslant 0.0001$) but not PICP ($p = 0.26$). In tumor specific analysis, prostate cancer follows a different pattern of bone remodeling with excessive increase of osteoblast function, as reflected by elevated values of OPG ($p = 0.0034$) and bALP ($p = 0.002$) but without any significant change of bone resorption markers such as sRANKL, TRACP-5b, CTX and OPN.
Conclusions: These results suggest that enhanced osteoclastic activity in patients with solid tumors metastatic to bone is mediated through severe disruption of the sRANKL/OPG axis in bone marrow microenvironment. Breast and lung cancer seem to exert their osteolytic action through upregulation of the sRANKL/OPG system, whereas prostate cancer seems to provoke profound elevation of OPG levels only, resulting in increased osteoblastic activity.

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Upregulated claudin-1 and downregulated claudin-3 and claudin-4 expression differentiates human hepatocellular carcinoma from colorectal liver metastasis

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Background: Tight junction proteins as claudins, occludin and JAM or peripheral zonula occludens (ZO) proteins are widely implicated in carcinogenesis. Claudins (1–24) have been recently identified as integral proteins of tight junction strands. Claudin-4 has not been found in normal hepatocytes or bile duct epithelium. Further, downregulated claudin-1 is associated with poor prognosis of colon carcinoma. Claudin-3 and -4 can function as the receptor of the Clostridium perfringens enterotoxin. Aim: The objective was to characterize the expression of claudins 1–4 and -7 in human HCCs and colorectal liver metastases compared with corresponding nontumorous and normal liver samples.
Material and methods: 19 human hepatocellular carcinoma (HCC) and 12 colon metastasis samples were examined by real-time RT-PCR and immunohistochemistry for expression of claudins-1–4 and -7. Relative quantification utilized GAPDH and beta-actin as internal control genes. The HCC samples did not show nuclear staining for beta-catenin indicating activation of the wnt pathway.
Results: Claudin-4 in HCCs was found downregulated 9.7 folds and 43 folds compared with normal livers and metastases, respectively. Claudin-3 in HCCs showed 5.5 fold downregulation in comparison to normal liver, however, there was 11 fold expressional downregulation compared with the metastases. Claudin-1 on the other hand showed 3.3 fold mRNA upregulation in HCCs compared with metastases. Immunohistochemistry predominantly detected membranous staining pattern and confirmed RNA expression data. Western blot analysis showed no expression of claudin-4 in HCC, however, immnohistochemistry detected the presence of claudin-4 on the bile ducts in nontumorous tissue.
Conclusion: Taken together, claudin-3 and -4 expressions were markedly downregulated in HCCs contrary to metastases, while claudin-1 showed higher expression in primary HCCs. Claudin-4 expression was localized to the bile ducts. These findings might help to differenciate hepatocellular carcinoma from colorectal metastasis. Biological role and significance of upregulated claudin-1 and downregulated claudin-4 in the progression of HCC should be elucidated.
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Oral presentations (Mon, 31 Oct, 9.15–11.15)
Polymorphisms as predictive markers

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A single nucleotide polymorphism in the CCND1 gene is associated with decreased risk of developing metastases in female breast cancer patients

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Background: One of the key regulators of the G1 phase in the cell cycle is Cyclin D1 (CCND1). In addition, CCND1 is an important factor in tumor development and progression. A common 870 G > A polymorphism in the gene for CCND1 has been linked to alternative splicing and cancer susceptibility. The objective of this study was to evaluate the role of this polymorphism for breast cancer metastazing.
Material and methods: A retrospective analysis including 500 female breast cancer patients was performed. In a Cox regression model including CCND1 870 A carriage, age at diagnosis, primary presence of regional

lymph node metastasis, primary tumor size larger than 2 cm, and estrogen and/or progesterone receptor negativity in the primary tumor the effect of the CCND1 genotype on metastasis-free survival time was estimated.
Results: The age of the patients at the time of diagnosis was between 28 and 84 years, with a mean age of 57 ± 11 years. Median metastasis-free survival time was 120 months (95%CI: 106–134). In the subgroup of 302 patients with stage III-IV breast cancer, 250 (82.8%) patients developed metastases in the time between diagnosis and study entry, whereas 52 (17.2%) patients remained free of metastases. The CCND1 870_AA genotype was found more frequently among patients without metastases (44.2%) than among those with metastases (24.8%; chi^2 test, p = 0.005). In a logistic regression model including age at diagnosis and estrogen and/or progesterone receptor negativity in the primary tumor as potential confounders, the 870_AA genotype was still significantly associated with metastasis risk (odds ratio 0.43, 95%CI: 0.23–0.83; p = 0.010).
Conclusions: Our data support the hypothesis that the CCND1 870 G > A polymorphism is associated with metastasis-free survival time in patients with breast cancer.

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Association of the A870G cyclin D1 gene polymorphism with genetic susceptibility to nasopharyngeal carcinoma

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Background: Nasopharyngeal cancer (NPC) is a human malignancy consistently associated with EBV. However, the etiology of NPC is complex and multifactorial, and exposure to non-viral carcinogens and genetic predisposititon are other crucial etiological factors. Cyclin D1 (CCND1) is a key regulator of the G1/S phase of the cell cycle and its altered activity is associated with the development of several human cancers, including Squamous Cell Carcinoma of the Head and Neck.
Material and methods: We analysed the A870G *CCND1* polymorphism by PCR-RFLP in 281 individuals including, 94 cases with NPC and 187 healthy individuals.
Results: Our results indicate that individuals carrying two G-alleles have a 2.17-fold increase in the risk for the development of NPC (OR = 2.17, 95%CI: 1.19–3.98, *P* = 0.016). Age-adjusted logistic regression analysis confirmed the association between the presence of the GG *CCND1* genotype and increased genetic susceptibility for the development of NPC (aOR = 2.14, 95%CI: 1.14–4.04, *P* = 0.018). Multivariate logistic regression analysis of the GG *CCND1* genotype (aOR = 2.06), male gender (aOR = 2.66) and age at diagnosis (aOR = 2.02) demonstrate an independent association between the *CCND1* GG genotype and the development of the undifferentiated histological type of nasopharyngeal carcinoma (UCNT). The proportion of cervical cancer cases attributable to the GG *CCND1* genotype was 14.76%.

Table 1: Multivariate analysis of the GG *CCND1* genotype, gender and age at diagnosis regarding the susceptibility to undifferentiated histological type of nasopharyngeal carcinoma (UCNT)

	*P**	aOR*	95%CI*
GG genotype	0.039	2.06	1.04–4.09
Age ⩾ 50	0.019	2.02	1.12–3.62
Male gender	0.002	2.66	1.45–4.86

**P*, aOR and 95%CI using logistic regression analysis.

Our data suggest that A870G CCND1 polymorphism is associated with the susceptibility to NPC and supports evidence for a site-specific prevalence of genetic alterations. These results may be important in the definition of a biological predictive profile for the development of NPC.

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Increased risk of cervical cancer associated with cyclin D1 gene A870G polymorphism

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Background: Human papillomavirus (HPV) play the major role in the etiology of cervical cancer. However, a complex correlation between viral and cellular genes is necessary for cell cycle control deregulation in the progression to invasive cervical cancer (ICC). Cyclin D1 is an important positive regulator of the G1/S phase of the cell cycle.
Material and methods: We analysed the A870G *CCND1* polymorphism by PCR-RFLP in the genomic DNA isolated from peripheral blood of 246 women including, 50 cases with high-grade squamous intraepithelial lesions of the cervix (HSIL), 93 with ICC and 103 healthy women. Statistical analysis was performed using the computer software SPSS for Windows (version 11.5). Chi-square analysis was used to compare categorical variables and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between *CCND1* genotypes and cervical cancer risk. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95%CI for the influence of *CCND1* genotypes in the risk of cervical cancer, with adjustment for age. We estimated the cumulative probabilities for developing cervical cancer (cumulative hazard function plots) by the Kaplan-Meier methodology.
Results: The GG genotype was associated with a significantly higher risk of ICC (OR = 3.20, 95%IC 1.55–7.41, *P* = 0.001). Furthermore, our results indicate a 3.7 higher risk for the development of HSIL in women carrying the GG *CCND1* genotype (OR = 3.67, 95%CI 1.45–9.31, *P* = 0.007). Age-adjusted logistic regression analysis confirmed the association between the presence of the GG *CCND1* genotype and increased genetic susceptibility for the development of cervical cancer. The proportion of cervical cancer cases attributable to the GG *CCND1* genotype was 17.26%. Furthermore, our results suggest that GG genotype of *CCND1* A870G polymorphism is associated with an earlier onset of cervical cancer (log rank test: *P* = 0.015) (Figure 1).

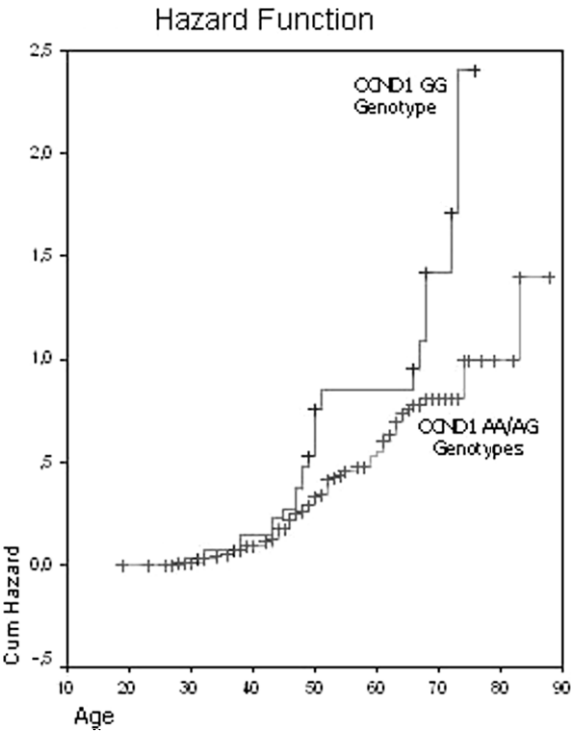

Fig. 1: Association of the A870G *CCND1* polymorphism and age of onset of cervical cancer. Cumulative hazard function plots by the Kaplan-Meier methodology and log rank test (*P* = 0.015).