SPANDIDOS PUBLICATIONS

Differential expression of osteocalcin during the metastatic progression of prostate cancer

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Abstract. Osteocalcin expression is restricted to osteoblasts and serum osteocalcin level is elevated in metastatic bone tumors including prostate tumors, which predominantly metastasizes to the bone and causes typical osteoblastic lesions. Previously, we have reported that osteocalcin RNA is widely expressed but incompletely spliced in the prostate including prostate tumors. Considering that many studies using osteocalcin-driven gene therapy have been conducted to treat hormone refractory metastatic tumors, detailed mechanisms controlling osteocalcin expression needs to be clarified. We aim to learn how osteocalcin expression is regulated during the metastatic process of prostate cancer. We applied assays of immunohistochemistry and RNA *in situ* hybridization in prostate tumors acquired from prostate (15) and metastatic sites, 13 from lymph node and 14 from bone. RT-PCR analysis in various cultured prostate cells was also performed. As predicted, osteocalcin RNA was highly expressed in most prostate epithelial cells of tumors, regardless of metastatic status of the tumor. However, osteocalcin protein was undetectable in tumors acquired from the primary site or lymph nodes whereas protein was highly expressed in the majority of bone-metastasized prostate tumors. RT-PCR analysis demonstrated that there was more completely spliced form of osteocalcin RNA present in bone-derived prostate cancer cells. Our data suggest that osteocalcin RNA was expressed but not completely spliced in non-bone environment, ultimately resulting in improper production of osteocalcin protein. This study explains why serum osteocalcin level is increased in patients with bone-metastasized prostate cancers. Yet, it remains to be clarified what regulates bone-specific osteocalcin RNA splicing in prostate tumors.

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Abbreviations: OC, osteocalcin; PCa, prostate cancer; IHC, immunohistochemistry; ISH, *in situ* hybridization

Key words: osteocalcin, prostate cancer, metastasis

Introduction

Human OC is composed of four exons and three introns and codes for 100 amino acid OC precursor, which further cleaves into a mature 51 amino acid peptide and constitutes 1-2% of the total protein in bone (1). OC functions at later stages of bone formation in the regulation of mineral deposition and turnover of bone. A number of studies showed that OC transcription is enhanced in mature osteoblasts while suppressed in early progenitors (2). Other than bone-related tissues, OC is synthesized by vascular smooth muscle cells and its mRNA is also expressed in megakaryocytes and peripheral blood platelets in rat, which possibly contribute to the OC levels in blood and the regulation of bone turnover (3).

Prostate cancer prevalently metastasizes to the bone and creates a common feature of osteosclerotic lesion with increased osteoblastic activity (4). It has been suggested that bone-metastasized PCa cells are osteomimetic and express genes and proteins which they normally do not express as bone-derived cells do. Since early 1990, several groups have observed that osteocalcin levels were significantly higher in patients with bone metastases whereas high levels were not observed in patients without bone metastases (5-8). High sensitivity and specificity levels of serum osteocalcin appear to be strongly correlated to metastatic bone involvement and disease relapse after hormone treatment. At the same time, Levedakou *et al* (9) have observed that OC was also highly expressed in primary prostatic carcinomas as compared to prostate cell lines derived from metastatic tumors, and to lymph node metastasis, suggesting that the loss of MGP expression may be associated with tumor progression and metastasis. Nevertheless, it is generally believed that OC

expression is increased by cis-acting transcription factors at OC promoter in androgen-independent prostate cancers, which are preferably metastasized to bone (10,11). On the other hand, we have previously observed that expression of OC in non-bone tissues, including normal prostate and prostatic cancer cells, mostly resulted from incomplete splicing event which may prevent proper formation of functional OC proteins (12).

In this study we hypothesized that OC expression may be altered during the course of prostate cancer progression, in terms of metastasis. To see the differential expression and tissue distribution of OC RNA and protein, assays of *in situ* hybridization (ISH) and immunohistochemistry (IHC) were performed in localized and metastatic human prostate tumors.

Materials and methods

Materials. Human prostate tumors were obtained from Department of Pathology at Indiana University. Tumors consisted of 15 local tumors, 13 lymph node-metastasized tumors, and 14 bone-metastasized tumors. Localized tumors were acquired from patients who underwent radical prostatectomy without history of metastasis. Lymph node-metastsized tumors were punch biopsy specimens. Bone-metastasized tumors were acquired though either biopsy or autopsy. All tumors were formalin-fixed and paraffin-embedded.

In situ hybridization (ISH). A set of primers (atttaggtgacacta tacactcctcgccctattggcc and taatacgactcactataggggccaactcgtca cagtccgg) was used for developing digoxgenin-labeled riboprobes for OC as described before (12). Labeled riboprobes were synthesized by *in vitro* transcription of PCR-amplified cDNA using digoxigenin-RNA labeling kit (Roche Biochemicals). RNA ISH was performed on formalin-fixed paraffin-embedded human prostate tumor tissues. ISH was performed using 30 ng of probe for each slide. Probes include OC antisense probes, OC sense probes as negative controls, and ß-actin probes as positive controls. Briefly, slides were deparaffinized and washed in diethyl pyrocarbonate (DEPC)-treated PBS, followed by DEPC-PBS containing 100 mM glycine. After the sections were treated with proteinase K (50 μg/ml) (Roche Molecular Biochemicals) at 37˚C for 30 min, 0.25% acetic anhydride (Sigma) in 100 mM triethanolamine (v/v) was applied for 10 min. The slides were prehybridized with 5X SSC and 40% deionized formamide at 42˚C for 30 min followed by hybridizing with dig-labeled riboprobes in hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA) at 42˚C overnight. Slides were washed twice at 50˚C in 2X SSC/0.1% SDS, then in 1X SSC/0.1% SDS, and 0.1X SSC for 30 min at room temperature. Signals were then developed using digoxigenin detection method (13).

Immunohistochemistry (IHC). The tissue distribution of OC protein was determined by immunohistochemical staining in fomalin-fixed and paraffin-embedded tissues. After deparaffinzing tissues, slides were microwaved for 10 min three times in 10 mM citrate buffer (pH 6.0) to retrieve antigen. Then, endogenous peroxidase was removed by the treatment of tissues with 0.3% H_2O_2 followed by avidin-biotin blocking. To inhibit non-specific binding of antibodies, slides were treated with 3% normal goat serum before the incubation of slides with monoclonal anti-OC antibodies (Takara Shuzo Co., OC4-30) overnight. Then, signals were amplified by horseradish peroxidase-DAB detection method.

RT-PCR analysis. Human osteosarcoma MG63 cells and various prostate cancer cell lines were used for the expression of OC RNA. Prostate cancer cells were LNCaP, C4-2, PC3, and MDAPCa2b as well as RWPE normal prostate cells. Cells were routinely cultured in RPMI media supplemented with 5% FBS at 37˚C in an atmosphere of 5% CO_2 before total RNAs were extracted. Total RNAs from each cell line were extracted by using Ultraspec RNA isolation system (Biotecx Laboratories) followed by RNase-free DNase (Promega Corp.) treatment to be free of the possible genomic DNA contamination. Total RNA (1 μg) with 100 pM d(T)20 was preheated at 72˚C for 5 min and reverse transcribed with MMTV RT (Invitrogen) at 42˚C for 1 h.

A set of OC primers, gaattcatgagagccctcacactcgcc and ctagactagaccgggccgagaagcgccgataggc, was used. Primers for ß-actin were used to show the equal loading (5'-gcacca caccttctacaatgagc-3', 5'-tagcacagcctggatagcaacg-3'). PCR was performed in a 50 μl solution containing 5 μl of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.2 mM dNTP mixture, 1 μl template DNA, 2 mM $MgCl_2$, 0.2 μM of each primer, and 2.5 units of Taq DNA polymerase (Invitrogen). Cycling parameters were: an initial denaturation at 94˚C for 2 min followed by 35 cycles with each cycle at 94˚C for 30 sec, at 63˚C for 30 sec, and at 72˚C for 2 min and the last extension at 72˚C for 7 min. PCR products (5 μl) were separated by 1% agarose gel electrophoresis.

Results

Expression of OC protein is limited to prostate tumor cells metastasized to bone marrow. For the localization of OC, patient samples were acquired through Indiana University. Samples were collected by either biopsy or autopsy. Biopsy samples of bone-metastasized PCa was obtained by bone marrow aspiration followed by cytospin. We have previously shown that OC RNA was abundantly expressed in the normal and cancerous prostate (12). Using RNA ISH, OC was mostly localized to epithelial cells of prostate tumors with moderate expression in stromal cells. However, most of OC RNA expressed in prostate tumors was incompletely spliced forms of OC containing one or more introns, resulting in abnormal expression of proteins. In this study, we used a pool of prostate tumors sorted by metastatic progression to evaluate expression of OC RNA and protein by ISH and IHC, respectively.

To support our hypothesis that OC splicing event is altered during metastatic process of prostate cancer, first, we investigated expression of OC RNA and protein in LNCaP and C42 prostate cancer cells using ISH and IHC (Fig. 1). LNCaP and C42 cells were previously demonstrated to mostly express incompletely spliced form of OC RNA (12). As we predicted, expression of OC RNA was abundant

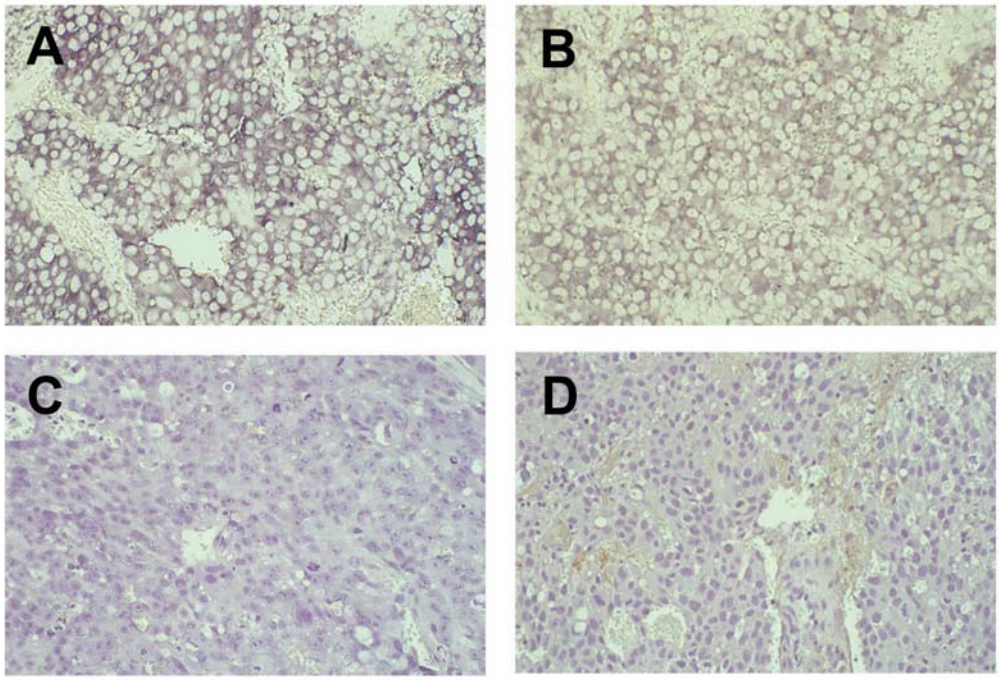

Figure 1. Expression of OC RNA and protein in xenograft tumors. LNCaP and C42 cells were injected into subdermis of nude mice to generate xenograft tumors. Tumors are retrieved from the animals and processed to generate histological samples. Slides then underwent ISH and immunohistochemical procedures. C and D were counterstained with hematoxyline.

in both cell types (Fig. 1A and B) while proteins are scant (Fig. 1C and D). This result demonstrates that our approach to detect both RNA and protein molecules can be adequately applied to test our hypothesis in tumors. Further studies have been done on prostate tumors of various metastatic grades (Fig. 2). By ISH, OC riboprobes were hybridized to its counterparts both in epithelial cells and in stromal cells of all prostate tumors, regardless of tumor origin (Fig. 2D-F). In localized tumors, OC RNA expression was high in most of the tumor epithelial cells whereas the OC level was very low in stromal cells (Fig. 2D). In tumors metastasized to lymph node and bone, OC RNA was detectable in prostate tumor cells in corresponding organs (arrows in Fig. 2D and F, respectively). OC probes were also highly reactive to osteoblasts present along the bony trabeculae (arrowheads in Fig. 2F). Some isolated tumor cells from bone marrow aspirates were also highly OC positive (arrow in Fig. 2Ia). OC RNA was non-reactive to OC sense probes in three different origins of tumors (Fig. 2G-Ib). Serial sections of prostate tumors were used to immunolocalize OC protein using anti-OC monoclonal antibodies. However, OC proteins were not detectable in tumor cells from either localized tumors (Fig. 2J) or lymph node-metastasized tumors (Fig. 2K). As we reported earlier (12), no or low expression of OC in these subsets of tumors were possibly due to the incomplete splicing event of OC. Surprisingly, OC protein was highly expressed in bone-metastasized prostate tumor cells (Fig. 2L). OC was not detectable in any of the tumors by using normal mouse IgG as a negative control (Fig. 2M-O).

Fig. 3 summarizes the staining score of OC RNA and protein by ISH and IHC, respectively. Scores were given as follows: 1, no staining; 2, weak and focal staining; 3 moderate and dispersed staining; 4, strong and extensive staining. Each score was statistically evaluated as mean ± standard deviation and shown as a bar graph. Overall, most OC RNA was moderately expressed in prostate tumors regardless of the origin of prostate tumors. However, OC protein was not detectable in localized and lymph node metastasized tumors while bone-metastasized tumors express moderate level of OC protein. These results suggest that transcriptional activity of OC is high in most of prostate tumors regardless of metastatic status but OC translation is somehow disrupted in majority of tumors except the ones in bone environment.

Bone environment affects the expression of OC protein. To demonstrate that expression of OC protein in bone-metastasized prostate tumors is not due to the sequestration of OC from nearby bone cells, we have selected several prostate cancer cells, including PC3 and MDA PCa 2b cells, bone-metastasized prostate cancer cells. LNCaP and C42 prostate cancer cells are derived from lymph nodes. RWPE cells are immortalized prostate cells. Extracted total RNA from cells and tissues were analyzed by RT-PCR using primers with its products encompassing exon 1 to exon 4 of OC gene as described (12). As shown in Fig. 4, RNAs from MG63 osteosarcoma cells exclusively express completely spiced form of OC, while RWPE, LNCaP, and C42 cells express variant size of OC, resulting from incomplete splicing event of OC gene. However, PC3 and MDA PCa 2b cells, bone-metastasized prostate cancer cells, express significantly higher amounts of completely spliced form of OC compared to non-bone-derived prostate cells. This result suggests that bone-metastasized prostate cancer cells acquire new phenomenon to completely splice OC RNA, resulting in proper production of OC protein. The result also leads us to predict that bone tissues may provide the proper

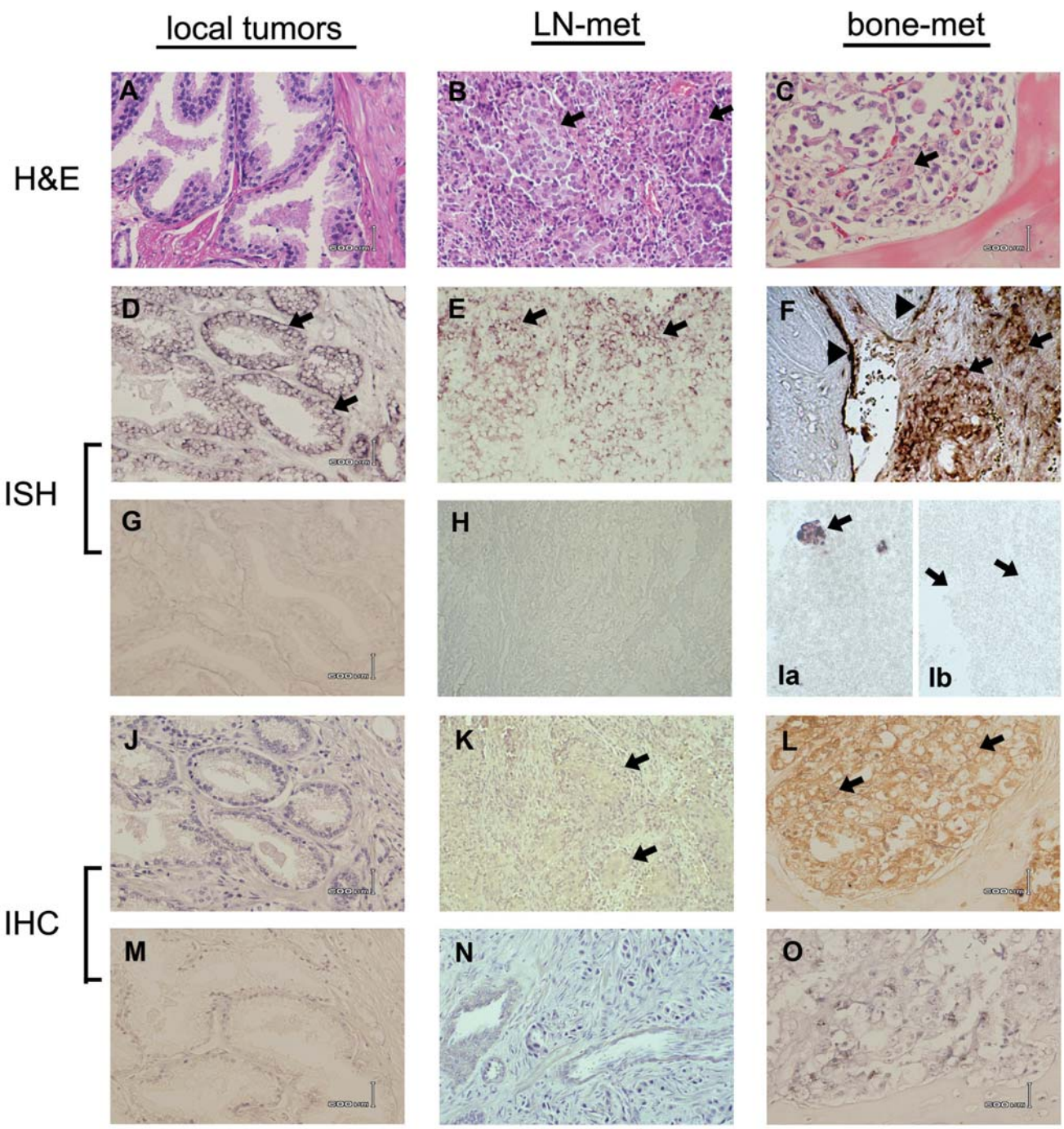

Figure 2. Expression of OC RNA and protein in prostate cancer tumors with various metastatic stage. Tissues with different metastatic origin were processed for detecting OC RNA and protein by ISH and IHC, respectively. (A-C), H&E stained; (D-I), OC RNA expression by ISH (D-F and Ia with antisense probes, G-H and Ib with sense probes); (J-O), OC protein expression by IHC (J-L with anti-OC antibodies, M-O with normal IgG).

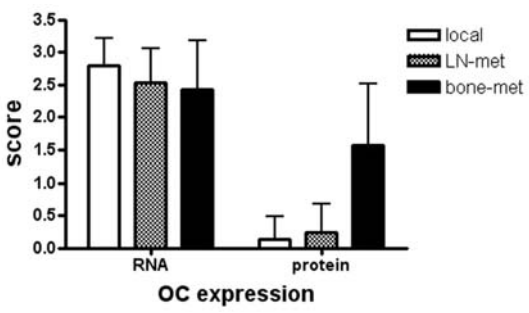

Figure 3. Statistical evaluation of OC RNA and protein expression in tumors. Scored OC expression is shown with mean ± standard deviation.

environment to complete OC splicing by turning on to express a bone tissue-specific splicing factor(s). Expression of OC by prostate cancer cells may stimulate the induction of osteoblastic lesions in the bone, the characteristic phenotype caused by prostate cancer. However, this hypothesis needs to be extensively tested to reveal the interaction between these two cell types.

Discussion

Osteocalcin is one of the most abundant non-collagenous bone matrix proteins expressed by osteoblasts and is thought

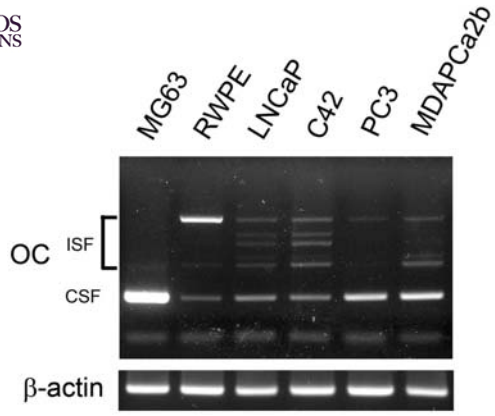

Figure 4. Expression of OC splice variants in cultured cells. Total RNA from cells were analyzed by RT-PCR. PCR products are shown in 1% agarose gel. ISF, incompletely spliced form; CSF, completely spliced form.

to play a role in osteoblast differentiation and mineralization at late stages of bone formation. Surprisingly, osteocalcin are also expressed by malignant prostate epithelial cells. Prostate tumors preferentially lodge in bone marrow and lead to typical osteoblastic legions. Interaction between prostate cancer cells and bone stromal cells is believed to initiate bone tropism of prostate cancer cells, resulting in the formation of osteoblastic lesions (5,14-16). Growth factors, including fibroblast growth factor 8, vascular endothelial growth factor, receptor activator of nuclear factor kappaB ligand with osteoprotegerin, and activation of extracellular signal-regulated protein kinase (ERK) and notch signaling are involved in this process (15,17-20). Besides, independent activation of ERK and Notch signaling can activate the expression of OC and generates osteoblastic lesion by prostate cancer cells in bone environment (20). Beta2-microglobulin enhances OC and bone sialoprotein gene expression in human prostate cancer cells by activating a cyclic AMP-dependent protein kinase A signaling pathway (21). Galectin-1 is also involved in the osteoblastic response, caused by prostate cancer cells metastasizing into bone, by affecting the matrix mineralization (14). At the same time, prostate-specific antigen produced by metastatic prostate cancer cells may participate in bone remodeling in favor of the development of osteoblastic metastases in the heterogeneous mixture of osteolytic and osteoblastic lesions (22).

Transcription of the murine OC gene is controlled by a complicated mechanism mediated by Runx2 (23,24), Msx2/Dlx5 homeodomain proteins (25), steroid hormone receptors including glucocorticoid receptor, estrogen receptor, vitamin D receptor, androgen (26-28), TLE co-repressor (29), HDAC3 histone deacetylase, CBP/p300 histone acetyltransferase (30), and SWI/SNF chromatin remodeling factors (31). Generally, OC transcriptional activity using mouse OC promoter is highly active in most prostate cancer cells, regardless of the status of androgen receptor and prostate-specific antigen. In fact, viral gene therapy using OC promoter to control the expression of toxic (therapeutic) gene in a tissue-specific manner is potentially effective to cure metastatic prostate cancers (32-36). We have previously demonstrated that expression of OC was also regulated by tissue-specific RNA splicing event. We identified incompletely spliced variants of human OC mRNA, existing dominantly in non-osseous organs. Most non-osseous tissues expressed transcripts with higher molecular weight, prominent in ovary, kidney, pancreas, spleen, thymus, prostate, and testis, than the expected size of OC mRNA as seen in bone marrow. Further analyses identified up to six OC transcripts in most tissues tested except bone marrow and most RNA variants contained one or more introns. MG63, an osteoblastic osteosarcoma cell, expressed only the completely-spliced form of OC, whereas incompletely spliced RNA was dominant in most prostate tumor cells (12). In this study, we investigated the presence of OC splicing variants during the course of metastatic process of prostate cancer. As expected, most localized tumors mainly express incompletely spliced forms of OC whereas bone-metastasized tumors acquire completely spliced form of OC and thereby mature form of OC protein. In contrast, prostate tumors metastasized to lymph nodes expressed high level of incomplete form of OC, but did not express OC proteins. Further RT-PCR analysis showed that all prostate cancer cells acquired from bone express significantly higher level of completely spliced form of OC compared to normal prostate cell and tumor cells from lymph nodes. This alternative RNA splicing event of OC may contribute to the acquisition of new osteoblastic activity of prostate cancer cells in bone environment. On the other hand, OC splice variants may provide tropism of prostate cancer cells to the bone, namely metastatic potential to the bone.

In conclusion, the expression of OC is known to be involved in the progression of prostate cancer metastasis and, in fact, its high serum level is associated with metastatic tumors (5-8). In this study, we demonstrated that prostate tumors express OC RNA but not much OC protein due to incomplete splicing process of OC pre-RNA. A similar result was observed in lymph node-metastasized tumors. Bone-metastasized prostate tumors, however, produce more properly-spliced OC RNA transcripts, which code for functional OC protein. With increasing usage of OC promoter based therapeutics to treat patients with bone-metastasized prostate cancer, the splicing phenomenon of OC and its biological role(s) in bone-metastasized prostate cancer cells need further investigation.

Acknowledgements

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