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BMP-7 expression is downregulated in human clear cell renal carcinoma

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ABSTRACT

Recent studies demonstrated that the expression pattern of bone morphogenetic protein 7 (BMP-7) is altered in different tumors. We determined expression of BMP-7 in human clear cell renal carcinoma (CCRC).

Samples from cancer and corresponding healthy tissue were obtained from 20 patients who underwent nephrectomy for CCRC. Expression of BMP-7 mRNA was determined by RT-PCR and protein expression was analyzed by immunohistochemistry.

RT-PCR showed strong downregulation of BMP-7 mRNA in cancer tissue. Immunohistochemistry revealed expression of BMP-7 in normal renal tissue, with almost complete loss of BMP-7 expression in malignant cells of 6 patients (30%). After 3 years of follow-up, 5 out of 6 patients with high BMP-7 mRNA expression were alive and disease-free, compared to 9 out of 14 patients with low BMP-7 mRNA expression.

BMP-7 mRNA and protein expression were downregulated in CCRC. Further prospective studies are needed to characterize the role of BMP-7 in human CCRC.

INTRODUCTION

Bone morphogenetic proteins belong to the transforming growth factor β (TGF-β) superfamily (1). Although first identified by their capacity to promote bone formation (2), they were found to play important roles in prenatal body patterning and morphogenesis, while during the postnatal life they may be involved in pathogenesis of different diseases (3,4). Bone morphogenetic protein-7 (BMP-7) is a 35-kDa homodimeric protein, predominantly synthesized in the kidney (5). BMP-7 knock-out mice die during the first postnatal day from uremia due to renal hypoplasia (6,7). In postnatal life, BMP-7 was found to be important in preservation of kidney structure and function and amelioration of injury. It inhibits tubular cell dedifferentiation, mesenchyme transformation, apoptosis, and protects kidney from diabetic nephropathy, acute and chronic renal failure (8-11). Dysregulation of BMP signaling has been suggested in carcinogenesis (12-18).

The clinicopathological significance of BMP-7 in human clear cell renal carcinoma has not been fully elucidated. We investigated BMP-7 mRNA and protein expression in samples of renal clear cell carcinoma and determined their prognostic significance after 3 years of follow-up.

METHODS

Tissue samples

Tissue samples were obtained at the Department of Urology, University Hospital Zagreb, Zagreb, Croatia, from 25 consecutive patients who underwent nephrectomy for renal cancer. Investigations were approved by the hospital ethic committee. Besides the abdominal multi-slice computed tomography, all patients underwent bone scan and chest X-ray to exclude disease dissemination before surgery. Tumor samples and corresponding healthy parts taken from the normal tissue located as far as possible from the tumor site were frozen in liquid nitrogen immediately after surgical resection, and were kept at -90°C until RNA extraction. Out of 25 tumor samples, there were 20 clear cell carcinomas that were further processed and these patients were included in investigation. Patients were followed-up for at least 3 years after surgery.

Quantitative Real-time RT-PCR

Messenger RNA (mRNA) was isolated from normal and cancer renal tissue using Quick PrepmRNA purification kit (GE Healthcare, UK) according to the manufacturer's instructions. The quantity and quality of mRNA was guaranteed by manufacturer.

Real-time RT-PCR was performed with a Light Cycler (Roche Applied Science, USA). Three micrograms of mRNA was reverse transcribed in reaction volume of 20 µl using FirstStrand cDNA synthesis kit (Amersham Biosciences, UK). The product was diluted to a volume of 50 µl, and 5 µl aliquots were used as templates for amplification using LC FastStart DNA Master SYBR Green I (Roche, USA) and gene-specific primers for BMP-7. Specific primers for each gene transcript were designed using previously published primers (sequence checked using GeneBank) and checked as to whether they showed a single peak in the dissociation curve. The primers for BMP-7 (276 bp) were: forward CAG CCT GCA AGA TAG CCA TT, and reverse GAG CAG GAA GAG ATC CGA TT (19).

For the analyses of the different genes, a separate master mix was made up for each primer pair and contained a final concentration of 1x LC FastStart Reaction Mix SYBR Green I (contains reaction buffer, LC FastStart Enzyme, dNTPs, SYBR Green I dye and 10 mM $MgCl_2$), 0,5 µM primers and 3 mM $MgCl_2$. Monitoring was done according to the manufacturer's instructions, as described previously (20). In brief, a master mixture was prepared on ice, containing 1 µl cDNA, 2 µl LC DNA Master SYBR Green Mix I mix, 50 ng

primers, and 2.4 μl 25 nmol/L $MgCl_2$. The amplification conditions of the 40 cycles consisted of denaturation at 95°C for 10 s, annealing at 65°C for 10 s, and extension at 72°C for 10 s. Obtained products were subjected to a temperature gradient from 68°C to 95°C at 0.1°C/s with continuous fluorescence monitoring to produce melting curves. All experiments were performed in triplicates. Quantification of housekeeping gene was performed with LC h-PBGD Housekeeping Gene Set according to the manufacturer's instructions (Roche, Germany).

Immunohistochemistry

Paraffin sections (3-4 μm) were deparaffinised in xylene and then rehydrated through graded alcohol. Endogenous peroxidase activity was blocked with 0,3 % hydrogen peroxide for 10 min. Anti – human BMP-7 monoclonal antibodies (R&D Systems, USA) were used for immunohistochemistry. Immunostaining was performed by the avidin biotin peroxidase complex method using LSAB+ kit (Dako, Glostrup, Denmark).

Evaluation of immunohistochemistry

Results of immunohistochemistry were interpreted using a light microscope. BMP-7 immunostaining was semiquantitatively evaluated for intensity (0, negative; 1 weak; 2, moderate; 3, strong staining).

Statistical analysis

SAS for Windows, version 9.1 (SAS Institute, Cary, USA) was used to perform statistical calculations. $P<0.05$ was considered statistically significant.

RESULTS

Patients's characteristics

There were 12 male and 8 female patients age ranging from 39 to 83 years (mean 63 years). The classic urological triad of haematuria, flank pain, and palpable mass was present in only one patient (5 %). Clinical findings at presentation are listed in Table 1.

Table 1.

Twelve patients had stage I, and 8 patients stage II of renal clear cell carcinoma according to Robson (21). Malignancy was an incidental finding on routine examination in 6 patients. Sixteen patients had one or more concomitant diseases including urolithiasis, diabetes mellitus, valvular heart disease and angina pectoris. One patient had previously been treated for malignant disease (acute myeloid leukaemia).

RT-PCR analysis of BMP-7mRNA expression in normal renal tissue versus renal clear cell carcinoma

Samples were initially obtained from 25 patients, but histological examinations demonstrated diagnosis of clear cell carcinoma in 20 patients, while oncocytomas and chromophobe tumors were excluded from investigation. Thus, forty kidney specimens obtained from 20 patients were evaluated for BMP-7 mRNA expression.

Under the conditions employed, BMP-7 mRNA expression was noted in all non-tumor renal tissue specimens and all renal clear cell cancer samples. The relative level of BMP-7 mRNA expression was determined with the reference to the internal PBDG control. Graph 1. shows ratio of BMP-7 mRNA expression normalized for PBGD expression in healthy and malignant tissue. Absolute numbers were transformed to logarithm scale because of huge differences in expression between the samples. It is evident that 17/20 patients had strongly downregulated BMP-7 mRNA in malignant tissue compared to healthy kidney parenchyma (up to 6.841 times lower BMP-7 mRNA expression normalized for PBGD). One patient exhibited only slightly higher level of BMP-7 mRNA expression in cancer compared to healthy tissue, while two patients had clear predominance of BMP-7 mRNA expression in malignant part of kidney (Graph 1). High ratio between healthy and malignant tissue was consequence of extreme downregulation of BMP-7 mRNA expression in malignant tissue.

Graph 1.

BMP-7 protein expression by immunohistochemistry

To confirm the above gene expression results demonstrating downregulated BMP-7 mRNA expression in malignant renal tissue, immunohistochemical analysis of the BMP-7 protein was performed.
Immunostaining for BMP-7 was stronger (count 1-2) in healthy tissue than in corresponding malignant tissue (count 0-1 as explained in Materials and Methods) in 6/20 patients (30 %). Interestingly, these were the only samples that exhibit positive staining for BMP-7 protein compared to BMP-7 mRNA expression in all samples. In healthy kidney tissue BMP-7 expression was dominantly localized to tubular cells (Figures 1 A and B).

Figures 1 A and B.

The level of expression of BMP-7 mRNA correlated with level of protein expression. Samples obtained from patients with high BMP-7 mRNA expression on RT-PCR examination demonstrated positive BMP-7 staining, while samples with low BMP-7 mRNA expression stayed negative after BMP-7 immunohistochemistry (count 0). Bone morphogenetic protein-7 expression was positive in both healthy and less intensively in malignant tissue of all patients with highly negative BMP-7 mRNA malignant-to-healthy tissue ratio. Samples from patients with positive BMP-7 mRNA malignant-to-healthy tissue ratio did not exhibit BMP-7 protein staining neither in healthy nor in malignant tissue.

Low BMP-7 mRNA expression may correlate with poor prognosis

After 3 years of follow-up, 5/6 (83.3 %) patients who exhibit BMP-7 staining on immunohistochemistry were alive and disease-free, compared to 9/14 (64.3 %) patients in whom immunohistochemical staining failed to demonstrate BMP-7 protein expression ($p=0.11$).
Bone morphogenetic protein-7 mRNA expression at nephrectomy was more accurate in predicting prognosis at 3 years of follow-up. Out of six patients with >-2 fold change in expression of BMP-7 mRNA from control (“high” expression group) 5 patients were disease-

free, and one patient died from heart attack. During the same period 9 out of 14 patients with lower BMP-7 mRNA expression remained alive and disease-free. Two patients from this group died from disseminated malignant disease, one of them had concomitant acute myeloid leukemia.

Comparison of survivors and deceased patients

Out of 6 patients with strongly positive BMP-7 staining in healthy tissue, 5 patients survived, and one died (Table 1.). Among the survivors, 16 patients had downregulated and only one increased BMP-7 mRNA expression (ratio between expression of BMP-7 mRNA in malignant and healthy tissue normalized for expression of PBGD), $p<0.05$. One deceased patient had increased and one downregulated BMP-7 mRNA expression (patient with leukemia), while the third exhibited only slightly higher level of BMP-7 mRNA expression in cancer compared to healthy tissue.

Discussion

To the best of our knowledge, this is the first paper dealing with BMP-7 mRNA expression in renal clear cell carcinoma. We found high BMP-7 mRNA expression in the healthy tissue relative to the renal clear cell carcinoma. Difference was less intensive although consistent at the level of protein expression. This is probably the reflection of different methodology which involved signal amplification in RT-PCR. Highly negative ratio between expression of BMP-7 mRNA in malignant and healthy tissue normalized for expression of PBGD was associated with favorable outcome at 3-year-follow-up. It resulted from high level of BMP-7 expression in healthy tissue and very low expression in malignant tissue. Kwak et al. have recently demonstrated BMP-7 expression in 34.1 % of renal cell carcinomas examined by immunohistochemical analysis of 185 cases, with expression rates being higher in chromofobe or papillary type than in clear cell type. They showed that patients with bone morphogenetic protein-7 expression have better disease-free survival than those without expression (22). Our study provided the same results of immunohistochemical analysis (30 % positive samples), but demonstrated that BMP-7 mRNA was expressed in both healthy and malignant tissue samples, with strong relationship between highly negative BMP-7 mRNA malignant-to-healthy tissue ratio and survival.

Action of BMP-7 is closely regulated not only by precise control of mRNA expression and protein processing, but is directed by influences of numerous agonists and antagonists. The importance of BMP-7 antagonist SOSTDC1 (sclerostin domain containing-1) in renal cancer has recently been investigated by Blish et al. They demonstrated decreased expression of SOSTDC1 in renal clear cell carcinoma. SOSTDC1 suppresses BMP-7 induced phosphorylation of Smad and Wnt signaling, and restoration of SOSTDC1 in renal clear cell carcinoma cell cultures suppresses proliferation (23). Noggin, a BMP antagonist suppresses growth of prostate cancer cells (24). On the other side, gremlin 1, another BMP-7 antagonist may enhance proliferation of various malignant cells (25). The story is further complicated with the finding of involvement of second messengers in development of neoplasia (26). Thus, Smad4, the main intracellular target of BMP signaling, was identified as tumor suppressor in pancreatic and intestinal cancer (27), and is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells (28). Numerous studies have linked members of BMP family, BMP antagonists, and BMP receptors to cancer (12-18). However, available data on involvement of BMP family members in cancerogenesis is conflicting. BMP-7 inhibits growth of prostate cancer cells, and

was strongly downregulated in malignant tissue compared to correspondent healthy prostatic tissue (29). Increased BMP-7 expression was found in various human cancers, including malignant melanoma, breast cancer, prostate cancer, ovarian cancer, osteosarcoma and colorectal cancer (12-18, 30, 31). To the contrast with previous results, BMP-7 mRNA expression was in our study significantly downregulated in cancerous tissue. Considering the differences in BMP-7 expression between various organs and its expression during embryonic development which is predominantly localized to kidneys, our results supports the hypothesis of protective role of BMP-7 in maintenance of adult kidney structure and function (8-11). Among the survivors, 16 patients had downregulated and only one increased BMP-7 mRNA expression (ratio between expression of BMP-7 mRNA in malignant and healthy tissue normalized for expression of PBGD), $p<0.05$. BMP-7 mRNA was downregulated in a patient who died from leukemia, but increased in two other deceased patients. Despite the small sample size, we may conclude that survivors had increased BMP-7 mRNA in the healthy tissue.

It is clear that activities and interactions between family members, their receptors and second messengers, as well as between agonists and antagonists are complex and may vary. Further investigations are needed to determine their exact role and utility as potential therapeutic targets in treatment of neoplasia.

In summary, our data demonstrate high level of expression of BMP-7 mRNA in normal renal parenchyma, with localization to the renal tubular cells. Furthermore, significant downregulation of BMP-7 mRNA expression was found in renal clear cell carcinoma. BMP-7 protein was also downregulated in renal clear cell cancer compared to adjacent healthy kidney parenchyma, but with significantly lower difference than at the gene level. BMP-7 may have protective role in maintaining structure and function of adult kidneys and its loss may lead to development of neoplasia. It is an intriguing possibility that persons with low level of BMP-7 mRNA expression may be vulnerable for development of renal neoplasia. Further prospective studies are needed to better characterize the role of BMP-7 in human clear cell carcinoma and to evaluate BMP-7 as a possible new prognostic factor in renal clear cell carcinoma.

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Graph 1. BMP-7 mRNA expression in tumor tissue relative to healthy tissue (logarithm scale). BMP-7 mRNA is normalized for PBGD expression. Patients with ≥-2 fold change in expression of BMP-7 mRNA from control belong to the «high» expression group.

Table 1. Demographics and clinopathological characteristics of patients with renal clear cell carcinoma included in the RT-PCR and immunohistochemical analysis of BMP-7 expression. Highly negative ratio between expression of BMP-7 mRNA in malignant and healthy tissue normalized for expression of PBGD * ($p<0.05$). Positive ratio between expression of BMP-7 mRNA in malignant and healthy tissue normalized for expression of PBGD #.

Variable	**Survivors (n=17)**	**Deceased (n=3)**
Age (mean, range)	46 (39-65)	67 (56-83)
Gender (female)	7	1
Size of tumor (cm), mean (range)	2.5 (2-3.4)	3.8 (2.7-4.8)
Stage I	12	0
Stage II	5	3
Haematuria	3	3
Flank pain	4	2
Palpable mass	0	1
Hypertension	10	0
Elevated sedimentation rate	10	2
Thrombocytopenia	0	1
Anaemia	0	1
Erythrocytosis	1	0
Elevated liver chemistries	1	1
Smoking	6	0
BMP-7 mRNA downregulated *	16	1
BMP-7 mRNA upregulated #	1	1
Positive healthy tissue staining for BMP-7	5	1

Figure 1. Immunostaining of BMP-7 was weak in normal renal tissue, but almost completely absent in the corresponding renal clear cell carcinoma tissue. Positive staining was mainly detected in the cytoplasm of tubular cells. Glomeruli were BMP-7 negative. (A) Healthy renal tissue, original magnification x 200, BMP-7 stain; (B) corresponding malignant renal clear cell carcinoma tissue, original magnification x 200, BMP-7 stain.