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Rapid report

# Ectopic expression of alkaline phosphatase in proximal tubular brush border membrane of human renal cell carcinoma

R. Prasad[a,*], S. Lambe[b], P. Kaler[a], S. Pathania[a], S. Kumar[a], S. Attri[a], S.K. Singh[b]

[a]*Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India*
[b]*Department of Urology, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India*

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**Abstract**

The present study was conducted to find out any alteration in the expression and activity of alkaline phosphatase in the brush border membrane (BBM) from renal cell carcinoma (RCC) in comparison to normal renal BBM. The specific activity of alkaline phosphatase was drastically reduced in homogenate as well as BBM from RCC kidney when compared to ALP activity in BBM of normal kidney. Kinetic studies revealed that diminished activity of alkaline phosphatase in BBM isolated from RCC was fraternized with decrease in maximal velocity ($V_{max}$) and increase in affinity constant ($K_m$) of the enzyme. SDS-PAGE studies showed that the BBM proteins having molecular weights ranging from 95 to 170 kDa were poorly expressed in RCC BBM in relative to normal kidney BBM. Incubation of SDS-PAGE gel with BCIP/NBT dye clearly showed that the expression of ALP in tumor renal BBM was markedly reduced as compared to normal kidney. Further, Western blot analysis using anti-alkaline phosphatase antibody also confirmed the reduced expression of ALP in tumor renal BBM. Lipid composition in reference to phospholipids, glycolipids and cholesterol in tumor renal BBM was altered to that of normal renal BBM, indicating alteration in membrane fluidity of tumor renal BBM.


*Keywords:* Alkaline phosphatase; Renal cell carcinoma; Brush border membrane; Lipid composition

## 1. Introduction

Renal cell carcinoma is the most common renal tumor accounting for approximately 3% of adult malignancies [1]. It attributes for 90% of all renal malignancies. Electron microscopy has revealed that human renal cell carcinoma inherits brush border membrane resembling those of convoluted tubules [2]. Further, on the basis of morphological, histological and immunochemical corroborations, it is conceived that RCC originates from proximal tubular epithelium of adult kidney. More recent data suggest that this applies to the more common clear cell variant of RCC, whereas other subtypes of RCC such as chromophobic appears to be derived from more distal elements of the nephron [3].

Alkaline phosphatase (ALP) is a family of cell surface glycoproteins that catalyzes the hydrolysis of phosphomonoesters with release of inorganic phosphate. Four major ALP isoenzymes have been characterized from humans each encoded by a separate gene [4]. Three of them are expressed in intestine, placenta and germ cells, whereas the fourth isoenzyme is highly expressed in liver, bone and kidney (L/B/K) and low in other tissues.

Determination of ALP activity is frequently used in clinical medicine. The cancer related research of the enzyme has been focused on the entopic or ectopic expression of ALP in several malignancies including brain tumors [5–8]. Strikingly, enzyme histochemistry in plastic embedded section of renal carcinoma showed poor reactions with alkaline phosphatase, which is suggestive of decreased activity of ALP in renal cell carcinoma [9]. An inverse

* Corresponding author. Tel.: +91 172 2747585 92x5178; fax: +91 172 2744401/2745078.
*E-mail address:* fateh1977@yahoo.com (R. Prasad).

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relationship has been found between cellular L/B/K ALP expression and aggressiveness in human osteosarcoma [10].

In order to investigate the role of L/B/K ALP in the pathophysiology of renal cell carcinoma, the present study was conducted to find out any alteration in the expression and activity of marker enzyme viz. alkaline phosphatase in the brush border membrane of renal cell carcinoma in comparison to normal kidney. Alterations in BBM lipid composition and fluidity may also play a role in the regulation of BBM bound enzyme activity [11–13]. Therefore, the attempts were also made to see any modification in the major lipid composition of BBM from renal cell carcinoma.

## 2. Materials and methods

### 2.1. Patients

The Ethics Committee of our institute approved this study and informed consent was obtained from patients. A total of 32 cases of renal cell carcinoma operated under Urology services of Nehru Hospital, Post Graduate Institute of Medical Education and Research, Chandigarh, were included in the study. All the patients underwent detailed clinical evaluation, biochemical and radiological investigation before surgery. Following nephrectomy, tissue samples were taken from grossly normal kidney and from grossly tumorous tissue separately. Staging and nuclear grading of the tumors was done according to TNM classification [14] and Fuhrman et al. [15].

### 2.2. Preparation of renal brush border membrane

Renal brush border membrane was prepared by differential centrifugation method as described by Prasad et al. [16]. 10% homogenate of the renal tissue was made in ice-cold 50 mM Mannitol buffered with 15 mM HEPES buffer (pH 6.9). $MnCl_2$ was added to a final concentration of 4 mM. The suspension was centrifuged at $4300\times g$ for 10 min. The supernatant so obtained was again centrifuged at $43000\times g$ for 20 min. The resulting pellet resuspended in 300 mM Mannitol buffered with 15 mM HEPES buffer (pH 7.2) represents the brush border membrane.

The purity of BBM was checked by assaying the marker enzyme of BBM i.e. alkaline phosphatase as described by Prasad [17]. The contamination of basolateral membrane (BLM) in isolated BBM was checked by assaying the marker enzyme of BLM, i.e., $Na^+/K^+$ ATPase by the method of Quigley and Gotterer [18].

### 2.3. SDS-PAGE

The BBM proteins were resolved on 10% SDS-PAGE by the method of Laemmli [19]. The bands were visualized by silver staining.

### 2.4. Detection of alkaline phosphatase

After electrophoresis, SDS-polyacrylamide gel was kept in double distilled water for 10 min and then incubated in 1X 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) dye, till the bands of alkaline phosphatase were visible. The gel was finally stored in 5% acetic acid.

### 2.5. Western blot analysis

50 μg of renal normal and tumor BBM proteins were resolved on 8% SDS-PAGE and transferred on to nitrocellulose membrane using transfer buffer (192 mM glycine/10 mM Tris/0.05% SDS/20% methanol) for 3 h at 100 V. The blot was blocked with 3% BSA in phosphate buffered saline for 12 h. Then, the blot was washed two times with 2% Tween 20 in PBS for 5 min each. The blot was then incubated with primary antibody anti-alkaline phosphatase at 1:500 dilution (Santa Cruz Biotechnology) for 3 h at room temperature. The blot was washed two times with 2% Tween 20 in PBS for 5 min each. The blot was incubated with secondary antibody (goat anti-rabbit IgG-HRP conjugated) for 1 h room temperature. The blot was washed two times with 2% Tween 20 in PBS for 5 min each. The colour was developed by incubating the blot in the developing solution (6 mg of diaminobenzidine in 10 ml PBS containing 10 μl 30% $H_2O_2$).

### 2.6. Analysis of BBM lipids

BBM lipids were extracted in chloroform: methanol by the method of Bligh and Dyer [20]. Phospholipids, glycolipids and cholesterol were estimated by the method of ESKO [21] and Zlatkis et al. [22].

### 2.7. Statistical analysis

In all the experiments, the data is expressed as mean ± S.D. of 32 patients for enzyme activities and 6 patients for lipid analysis. The data follows a normal Gaussian distribution. Therefore, the statistical significance analysis was performed by dependent Student's *t*-test using SPSS version-10 software.

## 3. Results

### 3.1. Patient characteristics

There were 20 males and 12 females. The patients were in the age group of 19–78 years with mean age of 53.75 years. The majority of the patients were in age group of 41–60 years. The commonest presenting complaints were hematuria and flank pain. All these cases were histologically proven clear cell carcinoma. The majority of patients (28 out of 32) had tumor in low stage (I and II) while 4 patients had tumor in high stage (III and IV). However, on the basis of

Table 1
Alkaline phosphatase and $Na^+/K^+$ ATPase activity in BBM and homogenate of kidney from tumor and normal group

| Enzyme | Specific activity (μmol/min/mg protein) | | | |
|---|---|---|---|---|
| | Group | Homogenate | BBM | Enrichment |
| Alkaline phosphatase | Normal | 0.163±0.04 | 1.175±0.24 | 7.46±1.86 |
| | Tumor | 0.033±0.01** | 0.230±0.07** | 7.06±1.98 |
| $Na^+/K^+$ ATPase | Normal | 0.515±0.12 | 0.170±0.02 | 0.21±0.04 |
| | Tumor | 0.417±0.09 | 0.146±0.01 | 0.16±0.25 |

All the values are mean±S.D. of 32 RCC patients. Statistical analysis was done by means of dependent Student's *t*-test.
** $P<0.001$ as compared to normal controls. Enrichment is the ratio of enzyme activity in BBM to homogenate.

nuclear grading of RCC, 31 cases were of low grade tumor and one case was of high grade tumor.

### 3.2. Effect of RCC on BBM preparation and enzyme activity

RCC had no significant effect on the purification of BBM isolated from the tumor kidney (Table 1). The enrichment for the BBM-specific marker enzyme viz. alkaline phosphatase was almost similar in normal and RCC groups (Table 1). The contamination with basolateral membrane was checked by assaying the marker enzyme $Na^+/K^+$ ATPase in the purified BBM and homogenate. There was negligible contamination with the basolateral membrane as suggested by a decrease in $Na^+/K^+$ ATPase activity in BBM preparation as compared to homogenate. However, there was no significant difference in $Na^+/K^+$ ATPase activity in homogenate and BBM from both the groups (Table 1). Further, kinetic analysis of ALP revealed that the maximal velocity ($V_{max}$) of the enzyme was significantly reduced in renal tumor BBM to that of normal renal BBM, whereas the affinity constant ($K_m$) was significantly increased in tumor renal BBM in comparison to that of normal renal BBM (Fig. 1). These findings suggest that decreased activity of ALP in tumor renal BBM could be due to both decreased number of ALP molecules and change in the topology of ALP molecule in the brush border membrane.

### 3.3. Alkaline phosphatase activity/expression in normal and tumor kidneys

A marked reduction ($P<0.001$) in alkaline phosphatase activity was observed in homogenate and isolated brush border membrane from tumor kidney to that of normal kidney. However, enrichment fold for ALP was similar in both the groups.

### 3.4. Effect of RCC on resolution pattern of renal BBM proteins on SDS-PAGE

Resolution pattern of BBM proteins from tumor and normal kidneys on 10% SDS-PAGE showed that high molecular weight proteins ranging between 95 and 170 kDa from tumor BBM were less intense as compared to normal BBM (Fig. 2A). This finding is suggestive of less expression of these proteins in BBM of tumor kidney. Subsequently, brush border membrane proteins were resolved on 8% SDS-PAGE. The gel was incubated in IX BCIP/NBT solution which specifically reacts with ALP. The

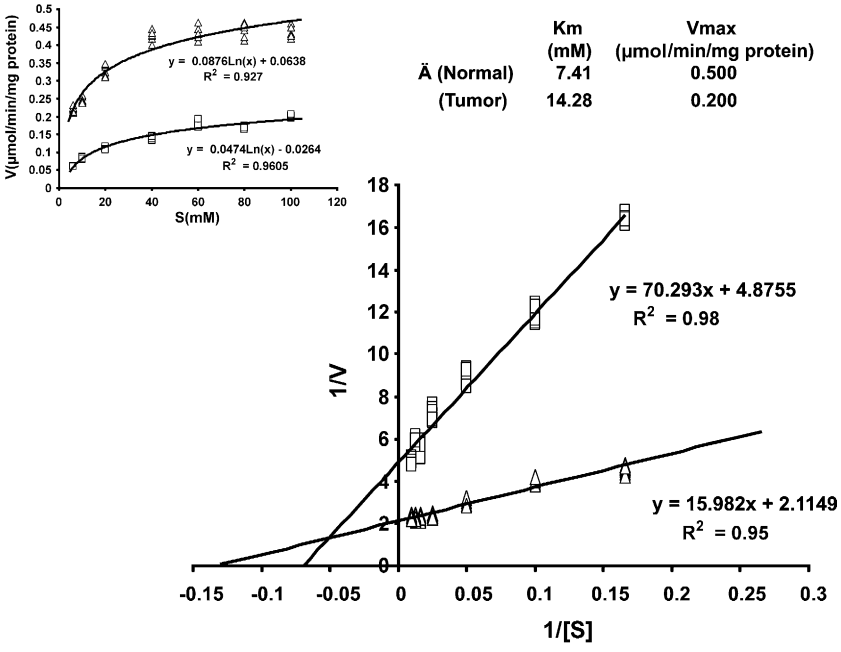


Fig. 1. Inset represents alkaline phosphatase activity in renal normal and tumor BBM as a function of varying substrate concentration. The best fit lines for both groups were plotted by nonlinear regression analysis. The data were rearranged to a linear form as Line Weaver Burk Plot. △ (Normal); □ (Tumor); $K_m$ (Michaelis–Menton constant) and $V_{max}$ (Maximal enzyme activity).

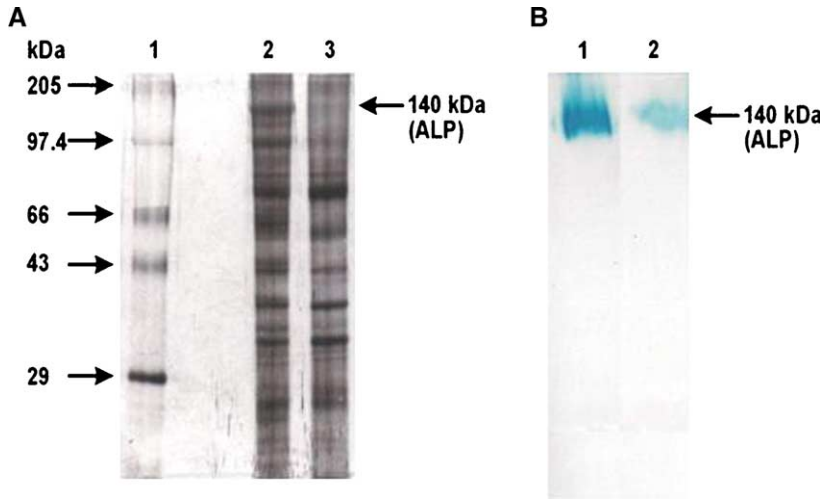


Fig. 2. Resolution pattern of BBM proteins on SDS-PAGE: (A) 10% gel stained with silver nitrate. Lane 1 represents the standard molecular weight markers, lane 2, normal renal BBM proteins and lane 3, tumor renal BBM proteins. (B) 8% gel incubated with BCIP/NBT dye. Lane 1 shows the alkaline phosphatase band developed for normal renal BBM and lane 2 shows the alkaline phosphatase band for tumor renal BBM.

prominent blue band of alkaline phosphatase was observed from normal BBM, whereas it was less intense in tumor BBM which further corroborates with the low expression of alkaline phosphatase in tumor BBM (Fig. 2B). Moreover, Western blot analysis confirmed that the expression of alkaline phosphatase was reduced in renal tumor BBM as compared to renal normal BBM (Fig. 3). This result is consistent with the BCIP/NBT staining.

### *3.5. Effect of RCC on BBM lipid composition*

BBM lipid composition was significantly altered in the RCC (Table 2). The levels of phospholipids and glycolipids were significantly higher in tumor renal BBM as compared to normal renal BBM. On the other hand, cholesterol content as well as cholesterol to phospholipid ratio in tumor renal BBM was significantly reduced when compared to normal renal BBM.

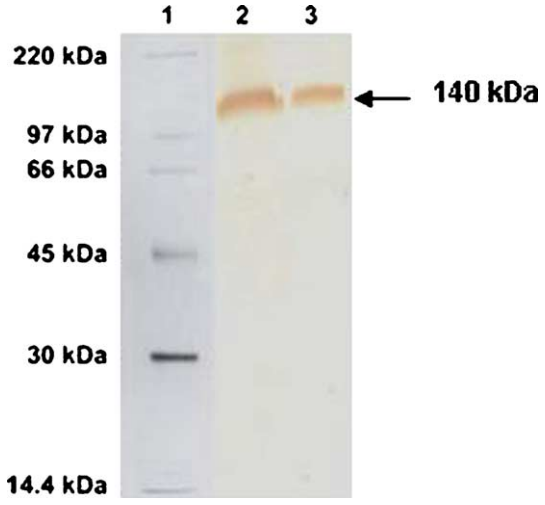


Fig. 3. Western blot analysis of alkaline phosphatase from normal and tumor renal BBM: BBM proteins from 8% SDS-PAGE were transferred to nitrocellulose membrane. Blot was probed with anti-alkaline phosphatase antibody and HRP-conjugated secondary antibody. Blot was visualized with enhanced chemiluminescence. Lane 1 shows the markers. Location of ALP is shown by arrow.

## 4. Discussion

Tumorigenesis is associated with altered cellular behaviour, disorganization of cytoskeleton and expression of novel proteins [23]. Certain proteins may be overexpressed in RCC while some may be decreased or absent. This alteration in expression of proteins in RCC could help in better understanding of molecular basis of pathophysiology of renal cell carcinoma and subsequently for the development of new tumor markers.

Decreased activity of ALP in tumor renal BBM confirms the weak reaction of kidney ALP observed during enzyme histochemistry [9]. Decreased activity of L/K/B ALP has also been reported in osteosarcoma [10]. Observed reduction in the activity of ALP may signify some tumor-specific changes at the molecular level which could be fraternized with various factors such as (I) reduction in expression of ALP mRNA (II) defect in posttranslational changes in ALP protein and subsequently defect in translocation of ALP protein into the brush border membrane (III) presence of mutations in the ALP gene (IV) modulation of brush border membrane fluidity due to alteration in BBM lipid composition. Modulation of maximal velocity and affinity constant of ALP in tumor BBM is suggestive of reduced turnover of ALP protein as well as alteration in membrane lipid microenvironment around the enzyme which is authenticated from the changes in BBM major lipid composition in the present study. It is a well known phenomenon that interactions between membrane lipids and proteins are important for regulation of membrane functions. Thus,

Table 2
Major lipid composition of brush border membrane from normal and tumor kidney tissue

| Group | Phospholipids (nmol/mg protein) | Glycolipids (nmol/mg protein) | Cholesterol (nmol/mg protein) | Ch/PLs |
|---|---|---|---|---|
| Normal | 100.03±14.70 | 26.31±6.00 | 95.80±15.4 | 0.958±0.066 |
| Tumor | 145.33±14.25* | 74.11±8.90* | 53.65±9.50* | 0.367±0.038** |

All the values are mean±S.D. of 6 observations. Statistical analysis was done by means of dependent Student's *t*-test. Ch—Cholesterol, PLs—Phospholipids.
* $P<0.05$ as compared to normal controls.
** $P<0.001$ as compared to normal controls.

alteration in the chemical composition and physical state of membrane lipids can modulate receptor, enzyme and transporter activity [11–13]. The increased expression of the long chain gangliosides in primary tumors is one of the factors associated with high metastatic potential [24]. Studies have also shown that ALP is anchored to the BBM via glycosylated phosphatidyl inositol [25]. The activity of alkaline phosphatase would therefore be expected to be strongly modulated by alterations in the composition and fluidity of the lipid micro environment. Nonetheless, measurement of detailed BBM lipid composition and fluidity is needed to support this notion. Cholesterol, because of its condensing effects on phospholipids, has shown a significant reduction in the fluidity of both artificial and biological membranes. Molar ratio of cholesterol to phospholipid was found to be two-fold lower in BBM isolated from tumor kidney as compared to normal kidney, implying increased fluidity of tumor renal BBM. Increased glycolipids in tumor BBM could also possibly modulate the ALP activity in tumor renal BBM since the BBM-glycosylated phosphatidyl inositol interaction results in a markedly increased lateral mobility of ALP molecule within the lipid bilayer [25,26]. In renal cell carcinoma, the levels of higher gangliosides are also correlated with degree of metastatic potential, and cell lines derived from metastatic deposits of RCC are characterized by high expression of disialogangliosides [24].

Although ALPs are present in many mammalian tissues, little is known about their physiological function in normal tissues and the role they play in the malignant processes. The L/B/K isoenzyme has been associated with bone calcification [27,28] and presence of mutations in ALP gene causes defective mineralization in hypophosphatasia [29]. Moreover, the enzyme has been suggested to act as tyrosine-specific phosphoprotein phosphatase and to be involved in the transport system of inorganic orthophosphate [30] and cellular migration [31]. The findings of Zucchini indicate a significant association between L/B/K ALP expression [10], activity and upregulation of cadherin 13 (CDH13) and caveolin1 (CAV1) genes related to cytoskeleton organization, cell–cell adhesion and cell growth [10]. However, the exact role of these potentially and functionally significant molecular abnormalities associated with RCC remains to be identified. An understanding of the reason underlying decreased expression/activity of ALP in renal tumor BBM may provide insight into the process of carcinogenesis and normal function of the enzyme along with a new tumor marker. Therefore, more work on proteomics and genomics in RCC is required for definitive conclusion.

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