Expression of neural cell adhesion molecule in renal cell carcinoma

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Neural cell adhesion molecule (NCAM) is important for cell migration and it could be expressed in some renal cell carcinoma (RCC). In recent decades, the incidence of RCC has been steadily rising by 2-4% each year. In this study NCAM expression and correlation with nuclear grade in different RCC were analyzed. We analyzed NCAM expression on 7 different RCC cell lines and 32 different RCC by immunohistochemistry, immunofluorescence, Western blot and FACS analysis. NCAM expression is detected in 6 cell lines and 16 RCC cases. NCAM-140 kDa isoform is expressed in different RCC and RCC cell lines. NCAM expression in non-invasive clear cell RCC is lower than in clear cell RCC with high nuclear grade. Expression of NCAM is not exclusive for specific RCC type, so NCAM cannot be used as a specific diagnostic marker for RCC. NCAM expression is in correlation with nuclear grade in clear cell RCC, suggesting that NCAM expression is involved in aggressive behavior and metastatic potential in RCC.

Key words: NCAM, tumor cell lines, RCC, nuclear grade.

INTRODUCTION

Cell adhesion molecules, including immunoglobulins, play important role in tumor genesis and metastatic cascade 1,2. Neural cell adhesion molecule (NCAM), a member of the large immunoglobulin superfamily (IgSF) of cell surface glycoproteins, is encoded by a single copy gene on chromosome 3. For the first time it was detected in neural system, where it has a role in Ca2+-dependent homophile and heterophile cell adhesion 3. Alternative splicing of its RNA results in three major isoforms, referred to as 120kDa, 140kDa and 180kDa molecules according to different molecular weight 4.

From clinical aspects NCAM is an important indicator of neuroendocrine differentiation of cells 5. Uninduced as well as induced foetal renal mesenchymal cells are well known to widely express NCAM as specific cell surface antigen up to the perinatal period during kidney development in humans and rats. Induced mesenchymal cells rapidly loose expression of NCAM after mesenchymal-epithelial transformation on immature tubular cells during further differentiation of the nephron 6.

Re-expression of NCAM is detected during postischemic regeneration in some tubules and interstitial fibrosis 7, as well as in renal cell carcinoma (RCC) in pediatric cases 8 and in RCC metastases in central nervous system (CNS) and adrenal glands 9.

In recent decades, the incidence of RCC has been steadily rising by 2-4% each year. In 2010, RCC was diagnosed in 90-95% of all renal neoplasms 10. RCC originates from the tubular structures of the kidney and is classified into four major histological types. Clear cell (cc) is the most common type, accounting for about 75-80% of all cases of RCC. Other types are papillary (10-15%), chromophobe (5%) and collecting duct (1%) RCC; fifth group presents unclassified RCC, less than 2%.

Aim of our study was detection of NCAM expression on different RCC type in tissue and on tumor cell lines, as well as correlation of NCAM expression with tumor nuclear grade.
MATERIAL AND METHOD

Renal tissues

Renal cell carcinoma (RCC) tissue was derived from 32 patients undergoing nephrectomy. One part of each renal specimen was processed routinely for light-microscopical evaluation. The other part of kidney samples was put into cell culture medium (RPMI 1640, Gibco) immediately after removal, snap frozen and stored in liquid nitrogen. Five µm thick frozen sections cut from each tissue were fixed in acetone for ten minutes, air-dried at room temperature for one hour and then used for immunohistochemistry and immunofluorescent analysis.

Renal cell lines

Seven RCC cell lines were used for analysis of NCAM expression. Four well characterized RCC lines (A-498, Caki-2, 786-0, ACHN) were obtained commercially from American Type Culture Collection (ATCC), and three cell lines (TW33, N43, NH) were derived from tumors of patients with RCC (UMG-Göttingen). The melanoma cell line FM3 was used as positive control (UMG-Göttingen). The cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal calf serum, penicillin and streptomycin and cultivated at 37°C with 5% CO2.

Antibodies

NCAM (Ancell) monoclonal antibody (mAb), clone ERIC-1 that detects the 120, 140 and 180 kDa isoforms of human NCAM was applied. This antibody was used for immunoblotting, FACS analysis, immunohistochemistry and double immunofluorescence staining.

Immunoperoxidase staining

In the present study immunoperoxidase staining was used on cryostat as well as on paraffin sections. Paraffin sections were treated by microwave for 3x3 min at 400W in citrate buffer (pH 6.0) after deparaffinization and dehyratation. After antigen retrieval samples were incubated with NCAM (dilution 1:100) for 1 hour at room temperature. For investigation of NCAM expression on renal tissues the EnVisionTM staining method (DAKO) was performed, followed by counterstaining with hemalaun (Merck). All experiments included sections stained with undiluted supernatant of the mAb W6/32HL (anti HLA-ABC heavy chain) as positive control, and of the mAb W6/32HK (inactive variant of W6/32HL) as negative control to exclude non-specific staining. Controls were also performed by omitting the first antibody and stained by the EnVisionTM method. The slides were evaluated using the light microscope BX53 (Olympus).

Immunofluorescence labeling of tissue

For the detection of NCAM+ cells in renal tissue indirect immunofluorescent staining was applied on frozen renal tissues. The tissue sections were incubated for 45 minutes with the mAb ERIC-1 (diluted 1:150) followed by Cy3TM-conjugated goat anti-mouse antibody diluted 1:700 (Dianova) as a second antibody. Negative controls were performed in all experiments by omitting the first antibody. The cell nuclei were identified by counterstaining with 1 µg/ml 4',6-diamidino-2-phenylindolyl-dihydrochloride (DAPI). The staining was visualized by fluorescence microscopy with CCD camera (Olympus). Digital pictures from every fluorescence channel were taken and superimposed for the specific antibody staining as well as for each negative control labeling using the software AnalySIS from Soft Imaging Systems (Olympus).
Flow-cytometry analysis

The extracellular expression of NCAM was assessed on RCC lines by BD FACSVerseTM flow cytometry (Becton Dickinson). FACS files were analyzed using the available software BD FACSuite (Becton Dickinson). The adherent RCC cells were harvested by incubation for 5 minutes with 0.05% (w/v) EDTA in PBS. Cells (1-3×10^5) were washed twice with washing buffer (0.1% w/v BSA in PBS). To avoid non-specific binding, cells were incubated for 10 minutes with human standard immunoglobulin Polyglobulin NTM (Bayer). Thereafter, cells were labeled with primary monoclonal antibody and FITC- or Cy2TM-conjugated goat anti-mouse IgG, dilution 1:500 (Dianova) as the secondary antibody. Two washing steps were performed before measuring the cells. As negative and positive controls, the mAbs W6/32HK and W6/32HL, respectively, were used.

Immunoblotting

Protein extracts from normal kidney tissue adjacent to tumour, RCC tissue and RCC cell lines were obtained by incubating the cells or tissue specimen for 30 minutes on ice in RIPA (Radio Immuno Precipitation Assay buffer) extraction buffer containing 1% Triton-X100, 1% NP40, 1 mM CaCl₂, 1 mM MgCl₂, 150 mM NaCl and 50 mM Tris-HCl pH 7.6. Proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose filters.
Non-specific protein binding sites were blocked with 5% milk powder, blocking reagent (Roche). The filters were incubated over night with primary antibodies against NCAM and subsequently washed for 15 minutes with PBS. Bound antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse (DAKO), followed by colorimetric reaction with the FastTM BCIP/NBT stem (Sigma).

RESULTS

Expression of NCAM seems to be present on RCC tissue regardless to histological type. RCCs with high nuclear grade have higher number of NCAM positive cells. In our tumor samples 20 cases were diagnosed as clear cell RCC, 6 as papillary RCC, chromophobe RCC was diagnosed only in 2 cases, while 4 cases had sarcomatoid variant of RCC (Table 1). In 32 analyzed renal carcinoma tissues, NCAM was present on tumor cells in half (16/32) of the cases. Diffuse, but strong staining on the cell surface of all tumor cells was detected in only 4 RCC (Figure 1a, b) whereas focal, moderate to strong expression of NCAM on large clusters of cells was present in 12 RCC. NCAM expression was not found in 2 samples of chromophobe RCC.

Semi-quantitative immunofluorescent analysis of clear cell RCC showed that NCAM expression in clear cell RCC is stronger in cases with higher nuclear grade (Figure 2a, b, c). In one tumor sample with normal kidney morphology, aberrant proximal tubular expression of NCAM was detected (Figure 2d). The related renal malignant tissue strongly revealed NCAM+ carcinoma cells.

Different RCC cell lines variably express NCAM on FACS analysis. From strongly positive to negative. We analyzed 6 RCC cell lines by flow cytometry for NCAM expression. Extracellular NCAM expression was detected on the next RCC cell lines: A-489, Caki-2, 786-0, TW33 and NH. The strongest NCAM expression was on 786-0, as well as on RCC cell lines A-498 and TW33. The cell line NH was obtained from RCC tissue where small focal NCAM expression was also detected by immunohistochemistry in situ, and this can explain small number of NCAM+ cells detected on FACS in this sample (Figure 3). The control melanoma cell line was also NCAM positive.

Detection of NCAM-140 kDa in renal carcinoma cell lines and tissue.
Western blot analysis of protein extracts obtained from renal carcinoma tissue which also showed positive signals for NCAM expression by immunohistochemistry was done. It revealed a NCAM-specific band of 140 kDa (Figure 4, lane 2- papillary RCC, line 3 - clear cell RCC, line 4- RCC cell line A-498; line 5 - RCC cell line 786-0), indicating that only the 140 kDa isoform of NCAM was present. In two of three analyzed renal cell carcinoma cell lines 140 kDa isoform of NCAM was also detected (Figure 4, line 4 - A-498 and line 5 - 786-0 cell line). NCAM-140 kDa isoform was not detected in one tumor sample with normal kidney morphology (Figure 4, line 1). No signal was observed by Western blotting in the ACHN cell line (not shown).

DISCUSSION

After completed organogenesis healthy tubules do not express NCAM16. Human RCCs seems to arise from a variety of specialized cells located along the nephron. Mainly clear cell, papillary and chromophobe RCC originate from proximal and distal tubules. These tubules originate from mesonephros after mesenchymal epithelial transformation during kidney development. Mesonephros strongly expresses NCAM, so re-expression of NCAM in tumor cell of RCC could be expected. Our results showed different level of NCAM expression in each RCC type as well as on different renal tumor cell line, suggesting that NCAM expression is present in RCC independently from histological type. NCAM expression was detected in clear cell, papillary and spatially in RCC with sarcomatoid variant. Intensity of NCAM expression is not even uniform within same RCC type. It had been showed that NCAM expression is proportional to tumor nuclear grade in clear cell RCC - stronger NCAM expression is detected in tumors with higher nuclear grade. Previous study reported that NCAM expression is stronger in RCC case which gave metastases in central nervous system (CNS) and adrenal glands. Also survival of patients with NCAM-expressing RCCs was lower than survival of other patients10. This finding is in correlation with our results and suggests than NCAM is expressed by a subgroup of aggressive RCCs, and it could be used to predict the potential biological behavior of clear cell RCC.

NCAM-140 kDa band on Western blot analysis was present in clear cell, papillary RCC and on tested renal tumor cell line. During CNS development NCAM-140 kDa isoform is responsible for cell migration and neurite growth through homophile interaction17. Expression of NCAM-140 in clear cell and papillary RCC and high NCAM expression by metastatic RCC cells invading CNS and adrenal glands10, two organs expressing high NCAM levels, indicates that tumor migration is favored by homophilic adhesion between tumor cells and host organ cells. Similar mechanisms have been reported to explain both the unusual sites of involvement in the case of NCAM-expressing T cell lymphomas18 and the lytic bone lesions of multiple myeloma19. On the other hand, for other tumors like pancreatic adenocarcinomas, reduced levels of NCAM expression were found to correlate with increased tumor malignancy20. Chromophobe RCC did not show NCAM expression in our study. Considering that only 2 samples of chromophobe RCC were analyzed, we can not say that lack of expression is characteristic of this chromophobe RCC.

Different renal tumor cell line also showed diffuse NCAM expression. Results obtained by FACS, Western blot, immunohistochemistry and immunofluorescent analyses showed that NCAM expression seems to be present on RCC tissue regardless of histological type, so NCAM could not be used as diagnostic marker in differential diagnosis of RCC. Nevertheless, correlation between NCAM expression and nuclear grade in clear cell RCC, suggests that NCAM expression is involved in aggressive behavior and metastastatic potential of clear cell RCC.

CONCLUSION

Analyzed renal tumor cell lines and clear cell and papillary RCC tissue samples showed presence of NCAM-140 kDa isoform. Expression of NCAM is not characteristic for any particular histological RCC type. Thus, NCAM is
not a useful marker for differential diagnosis of RCC. However, NCAM expression is in correlation with nuclear grade in clear cell RCC, suggesting that NCAM expression is involved in aggressive behavior and metastatic potential of RCC.

**SUMMARY**

**ISPOLJAVANJE NEURALNOG ĆELIJSKOG ADHEZIONOG MOLEKULA U KARCINOMIMA BUBREŽNIH ĆELIJA**

Neuralni ćelijski adhezioni molekul prvi put je opisan u centralnom nervnom sistemu. U bubregu se ispoljava u toku embrionalnog i fetalnog razvoja, ali tokom sazreva

njega ubreg postepeno se gubi, i u neonatalnom i u kasnijem periodu se pojavljuje samo na retkim intersticijskim ćelijama, dok ga na tubulima nema.

Incidenca bubrežnih tumora je svake godine u porastu za 2-4 %. Tako je 2010 godine karcinom bubrežnih ćelija (KB) dijagnostikovan u oko 90% ukupnih bubrežnih neoplazmi. Postoji više tipova KB, svaki se odlikuje drugačijom patohistološkom slikom i različitim biološkim ponašanjem. U našoj studiji analizirana je ekspresija NCAM izoformi i korelacija NCAM ekspresije sa tipom KB i nuklearnim gradusom.

Ćelijeske linije KB, kao i tumorsko tkivo svetloćelijskog i papilarnog KB-a ispoljavaju specifičnu NCAM 140 kDa izoformu. Intenzitet NCAM ekspresije kod svetloćelijskog KB je u korelaciji sa povećanjem nuklearnog gradusa. S tim u vezi NCAM ukazuje na slabiju diferencijaciju KB-a, dok ga na tubulima nema.

Ključne reči: NCAM, karcinom bubrežnih ćelija (KB), nuklearni gradus

**REFERENCES**


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