Annexin II represents metastatic potential in clear-cell renal cell carcinoma

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Renal cell carcinomas (RCCs) account for ~3% of adult tumours. The RCCs comprise a heterogeneous group of tumours with distinct genetic backgrounds and different biological characteristics. The most common subtype of RCC is clear-cell RCC (ccRCC), which represents over 80% of RCCs (Srigley, 2004). The widespread use of non-invasive imaging techniques (ultrasonography, computed tomography and magnetic resonance imaging) for abdominal screening or unrelated disorders has led to a widespread use of non-invasive imaging techniques (ultrasonography, computed tomography and magnetic resonance imaging) for abdominal screening or unrelated disorders has led to a migration towards earlier stages at diagnosis, with increased numbers of asymptomatic small renal tumour being detected. These incidentally detected RCC have a more favourable prognosis than symptomatic RCC (Pantuck et al., 2000). The Tumor-Node-Metastasis (TNM) stage and nuclear grade are currently widely accepted prognostic factors (Shuch et al., 2006), however, some patients infrequently follow unexpected clinical course such as late recurrence. Thus, the identification of additional prognostic markers more strictly related to the intrinsic biological behaviour of RCC may be helpful to plan postoperative follow-up protocol in individual patient, as well as to prevent the unnecessary use of adjuvant therapy.

Recently, we succeeded in establishing two RCC cell lines, which were derived from a matched pair of a primary RCC and its adrenal metastasis (Ohno et al., 2008). Proteomic analysis showed that annexin A2 (ANX2) is one of the proteins that are differentially expressed between these cell lines. The ANX2, a 34–36 kDa protein, belongs to a family of Ca2+-dependent phospholipids and membrane-binding proteins. The ANX2 is involved in specific functions that depend on its intracellular localisation, such as DNA synthesis in the nucleus, protein transportation in the cytoplasm. Further, on the cell surface it functions as a receptor for tissue-type plasminogen activator and tenascin-C (Gerke et al, 2005). As for the role in cancer, it is reported that overexpression of ANX2 is associated with progression and metastasis in various types of cancer, including brain tumour (Reeves et al, 1992), pancreatic cancer (Diaz et al, 2001a; Esposito et al, 2006), gastric cancer (Emoto et al, 2001a) and colorectal cancer (Emoto et al, 2001b). Recently, an in vitro study of rat RCC showed an association between ANX2 overexpression and metastasis (Tanaka et al, 2004). In addition, another report showed that ANX2 expression correlated with higher nuclear grade of the tumour and poor clinical outcome, although the study included only 33 cases (Zimmermann et al, 2004). These findings imply that ANX2 may be involved in the metastatic process in RCC. Therefore, in this study, we investigated the ANX2 expression in primary tumours in the context of its prognostic significance in RCC and RCC metastases.

MATERIALS AND METHODS

Clinical samples

Fresh frozen surgical specimens and formalin-fixed paraffin-embedded tissues were obtained from patients with ccRCC who were treated at the Tokyo Medical University Hospital. The
informed consent for the use of the samples was obtained from each patient.

Fresh frozen surgical specimens of primary tumour tissue paired with normal renal cortical tissue were obtained from 18 patients, who were treated between January 2004 and February 2008, at the time of nephrectomy and stored at −80°C until use. A total of 10 patients had T1 tumours, two had T2 tumours and six had T3 tumours. One tumour was graded as 1; eight as grade 2; nine as grade 3 or 4. At the time of diagnosis, seven patients had metastatic disease, and one patient developed metastasis during follow-up. In addition, formalin-fixed paraffin-embedded tissues were obtained from 161 patients who were treated between September 1986 and September 2003, which included 154 primary cRCCs and 24 metastatic tumours of cRCC. The metastatic tissue samples were obtained by surgical resection or tumour biopsy. The sites of metastasis were as follows: bone (n = 5), lymph node (n = 3), adrenal gland (n = 3), skin (n = 3), lung (n = 2), brain (n = 2), parotid gland (n = 2), thyroid gland (n = 1), ovary (n = 1), gall bladder (n = 1), retroperitoneal tissue (n = 1) and soft tissue (n = 1). The samples included 15 matched pairs of primary tumours and their metastases. The mean age of these 161 patients (117 men and 44 women) at the time of diagnosis was 59 years (range, 24–81 years). A total of 154 patients had undergone radical nephrectomy at our hospital. Extended lymphadenectomy was not included in the routine procedure of nephrectomy, but regional lymph node dissection was performed if enlarged or palpable lymph nodes were recognised. No patient received irradiation or chemotherapy before surgery. The tumours were staged according to the 1997 TNM staging system: 79 were TNM stage I, 12 were stage II, 28 were stage III and 35 were stage IV. The histological types were determined according to the World Health Organization classification. Nuclear grading was performed according to the Fuhrman’s nuclear grading system: 48 tumours were graded as grade 1; 68 as grade 2; 38 as grade 3 or 4. All patients were followed-up with clinical and radiological examinations at regular intervals. Patients with metastatic disease received interferon-α or interleukin-2 therapy. At the last follow-up, 76 patients showed no evidence of disease, 29 patients were alive with metastases, 46 patients had died of cancer and 10 patients had deceased because of other events. The mean follow-up period was 89.9 months (range, 1–261 months).

**Real-time RT–PCR**

A total of 18 pairs of primary RCC and normal renal cortex were used in this analysis. Whole-tissue protein extracts were collected after sonication with 2 × sample buffer (containing 0.25 M Tris-HCl, 10% 2-Mercaptoethanol, 4% sodium dodecyl sulphate and 10% sucrose). The protein concentration was determined using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at 37°C. Immunoreactive proteins were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia) and LumiVision H (Taitco Co., Tokyo, Japan). Subsequently, the membranes were stripped off the first probe by using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and reprobed with mouse anti-human β-actin monoclonal antibody (dilution at 1:2000; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody used for anti-human β-actin antibody was anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (dilution at 1:1200, Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at room temperature. The membrane with bound antibodies was then incubated with anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution at 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The membrane with bound antibodies was then incubated with anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution at 1:2000, Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at 37°C. Immunoreactive proteins were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia) and LumiVision H (Taitco Co., Tokyo, Japan). Subsequently, the membranes were stripped off the first probe by using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and reprobed with mouse anti-human β-actin monoclonal antibody (dilution at 1:2000; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody used for anti-human β-actin antibody was anti-mouse horseradish peroxidase-conjugated IgG antibody (dilution at 1:2000, Amersham Pharmacia). Densitometric analysis was performed by using a LumiVision analyzer (Taitco Co., Tokyo, Japan). The ANX2 protein level was expressed with the relative ratio (RR), which was calculated by the following formula using signal intensity (SI) of ANX2 and β-actin.

\[
RR = \frac{SI_{\text{ANX2}}}{SI_{\beta-\text{actin}}}
\]

**Western blot analysis**

A total of 18 pairs of primary RCC and normal renal cortex were used in this analysis. Whole-tissue protein extracts were collected after sonication with 2 × sample buffer (containing 0.25 M Tris-HCl, 10% 2-Mercaptoethanol, 4% sodium dodecyl sulphate and 10% sucrose). The protein concentration was determined using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at 37°C. Immunoreactive proteins were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia) and LumiVision H (Taitco Co., Tokyo, Japan). Subsequently, the membranes were stripped off the first probe by using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and reprobed with mouse anti-human β-actin monoclonal antibody (dilution at 1:2000; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody used for anti-human β-actin antibody was anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (dilution at 1:2000, Amersham Pharmacia). Densitometric analysis was performed by using a LumiVision analyzer (Taitco Co., Tokyo, Japan). The ANX2 protein level was expressed with the relative ratio (RR), which was calculated by the following formula using signal intensity (SI) of ANX2 and β-actin.

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\]

**Immunohistochemistry**

The sections (4-μm-thick) from archival formalin-fixed paraffin-embedded tissues of a representative area of the surgical specimens, which included the highest nuclear grade cancer, were mounted on poly-L-lysine coated slides. They were deparaffinised with xylene and rehydrated through a graded alcohol series. Endogeneous peroxidase was blocked by incubation with 3% hydrogen peroxide for 20 min. Antigen retrieval (121°C for 10 min in 10 mM citrate buffer, pH 6.0) was then performed. Endogenous biotin was blocked by incubation with 0.01% biotin for 20 min at room temperature. After blocking non-specific conjugation with 1% casein, the slides were incubated for 60 min at room temperature with anti-annexin II rabbit polyclonal antibody (dilution at 1:50; Santa Cruz Biotechnology). Bound antibodies were detected by the avidin–biotin complex peroxidase method (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) and visualised with diaminobenzidine. The slides were counterstained in Harris’ hematoxylin, dehydrated and
mounted. Sections of benign prostate tissue were used as positive control. As negative control, the primary antibody was omitted and substituted with normal rabbit IgG. The ANX2 expression was considered to be positive if >10% of the cancer cells were clearly stained in each slide under microscopic observation.

Statistical analysis

All statistical analyses were performed using JMP Ver. 5.1 (SAS Institute Inc., Cary, NC, USA). The ANX2 gene by real-time RT–PCR and ANX2 protein expression by western blot were assessed using paired t-test. In immunohistochemical analysis, ANX2 expression was considered as a dichotomous variable (i.e., positive or negative) in all the statistical analyses. The correlation between ANX2 expression and other clinicopathological factors was assessed using a χ-square test, Fisher’s exact test and Student’s t-test. The metastasis-free and cancer-specific survival rates were estimated by the Kaplan–Meier method, and the differences between the curves were tested using the log-rank test. Metastasis-free time was calculated from the date of radical nephrectomy to the date of radiological detection of metastases. A multivariate analysis using the Cox proportional hazards regression model was used to test for independent prognostic values. P-values <0.05 were considered statistically significant.

RESULTS

Annexin II expression in primary RCC tissues: results of real-time RT–PCR and western blot analysis

A total of 18 pairs of primary kidney cancer and a corresponding normal renal cortex were analysed. The ANX2 was upregulated at

Figure 1 Annexin II (ANX2) expression in primary kidney cancer. (A) The ANX2 expression at mRNA level was analysed in 18 pairs of primary clear-cell renal cell carcinoma (T) and the corresponding normal renal cortex (N). The ANX2 was significantly upregulated in 14 primary tumours (P = 0.0086). (B) Western blot analysis showed ANX2 expression was higher in 14 of 18 primary tumours compared with the corresponding normal renal cortex; however, the difference was not significant. The relative ratio (RR) is calculated using signal intensity of ANX2 and β-actin. (C) Representative examples of ANX2 protein expression in primary kidney cancer. After normalising the signal intensity of ANX2, the ANX2 expression in each sample was evaluated on the basis of the ratio of the primary kidney cancer to the corresponding normal renal cortex. Patients 1 – 3 showed no evidence of metastasis, patient 4 developed metastasis 3 years after nephrectomy, and patients 5 and 6 showed metastasis at the time of diagnosis.
both mRNA and protein levels in 14 of 18 primary RCC tissues in comparison to the corresponding normal renal tissues; especially, the ΔCT value of tumour (mean ± s.d., $-3.07 \pm 0.71$) was significantly higher than those of the corresponding normal renal tissue (mean ± s.d., $-3.80 \pm 0.76$) ($P = 0.0086$) (Figure 1A and B).

Representative examples of ANX2 expression by western blot analysis are shown in Figure 1C. The primary kidney cancer (T) / the corresponding normal renal cortex (N) ratio of ANX2 expression was $0.33 - 5.73$ (mean ratio, 1.96). The mean T/N ratio in patients with metastasis (mean ± s.d., $2.62 \pm 0.44$) was more likely to be higher than that in those without metastasis (mean ± s.d., $1.44 \pm 0.39$) ($P = 0.0617$).

Annexin II expression in paraffin-embedded primary kidney cancer and its metastases: results of immunohistochemistry

Immunohistochemical analysis of 154 primary RCCs and 24 metastatic tumours was performed. The ANX2 staining was observed on the cell membranes or in the cytoplasm. The ANX2 staining was heterogenous in the tumour sections, and the staining intensity was relatively higher in the periphery of the tumour and around vessels (Figure 2A–D). Finally, 73 (47.4%) of the 154 primary RCCs and 21 (87.5%) of the 24 metastatic tumours were considered to be positive for ANX2. There was a significant
statistical difference in ANX2 positivity between a primary RCC and its metastasis \( (P = 0.00028) \). Among the 15 matched pairs of primary RCC and its metastasis, both components were positive for ANX2 in 12 pairs. Representative images of the metastatic tumours (lung, lymph node, bone and brain) are also shown in Figure 2E–H.

**Correlation between annexin II expression and clinicopathological factors**

Table 1 shows the correlation between ANX2 expression in primary RCC and clinicopathological factors in the 154 patients who underwent nephrectomy. The ANX2 expression was more frequent in tumours of a higher TNM stage \( (P = 0.00005) \) and microscopic venous invasion \( (P = 0.0007) \) were also associated with ANX2 positivity.

| Clinicopathological factor | ANX2 expression | \( P \)-value |
|----------------------------|-----------------|--------------|
| Age (years)                | 57.3 60.1       | NS           |
| Size (cm)                  | 5.3 6.9         | 0.0028       |
| Gender                     | 50 (44.6) 62 (55.4) | 0.001        |
| Male                       | 31 (73.8) 11 (26.2) |              |
| Female                     | 4 (33.3) 8 (66.7)       |              |
| TNM stage                  | 21 (26.6) <0.0001 |              |
| I                          | 58 (73.4)       |              |
| II                         | 4 (33.3) 8 (66.7) |              |
| III                        | 10 (35.7) 18 (64.3) |              |
| IV                         | 9 (25.7) 26 (74.3) |              |
| T stage                    | 29 (33.3) 12 (57.1) | 0.0006       |
| T1                         | 58 (66.7)       |              |
| T2                         | 9 (42.9) 12 (57.1) |              |
| T3                         | 13 (31.7) 28 (68.3) |              |
| T4                         | 1 (20.0) 4 (80.0)       |              |
| M stage                    | 23 (76.7) 12 (22.9) <0.0001 |
| 0                          | 74 (59.7) 50 (40.3) | 0.0003       |
| I                          | 7 (23.3) 23 (76.7) |              |
| Grade                      | 1 12 (31.6) 26 (68.4) <0.0001 |
| 1 or 2                     | 37 (77.1) 11 (22.9) |              |
| 2                          | 32 (47.1) 36 (52.9) |              |
| 3 or 4                     | 12 (31.6) 26 (68.4) |              |
| Microscopic venous invasion| 63 (60.0) 42 (40.0) 0.007 |
| Negative                   | 18 (36.7) 31 (63.3) |              |

**Prognostic significance of annexin II expression in RCC**

To assess the prognostic significance of ANX2 expression in RCC, we analysed cancer progression (metastasis) and survival in relation to ANX2 expression in primary tumour.

The relationship between clinicopathological factors and progression was analysed in 119 patients with stages 1–3 disease. A total of 41 patients developed metastases during their clinical course. The median time to radiological appearance of metastases was 50.5 months (range, 1.5–184 months). The following were the first recognised sites of metastasis: the lung in 26 cases, bone in 4 cases, liver in 1 case, adrenal gland in 1 case, retroperitoneal space in 4 cases and others in 5 cases. Metastasis-free rate was estimated using the Kaplan–Meier method. There was a significant inverse correlation between metastasis-free rate and the TNM stage \( (P = 0.0005) \) or nuclear grade \( (P = 0.0001) \) of the tumour. Microscopic venous invasion did not have significant influence on the development of metastasis. As for ANX2 expression, the metastasis-free rate in patients with ANX2-positive primary RCC was 63.0% over 5 years and 46.5% over 10 years; this rate was significantly lower than the 90.1% 5-year metastasis-free survival observed in patients with ANX2-negative primary RCC \( (P = 0.0036) \).

![Figure 3](https://example.com/f3.png)

**Figure 3** Metastasis-free survival rates in patients with stages 1–3 and the cancer-specific survival rates. The metastasis-free survival rates were estimated by the Kaplan–Meier method, and the differences between curves were tested using the log-rank test. (A) The metastasis-free survival rates in patients with stages 1–3 disease \( (n = 119) \). The metastasis-free time was calculated from the date of radical nephrectomy to the date of radiological detection of metastases. The metastasis-free survival rate of patients with ANX2-positive primary tumour was significantly lower than that of patients with ANX2-negative primary tumour \( (P < 0.0001) \). (B) Cancer-specific survival rates \( (n = 154) \). The survival rate of patients with ANX2-positive primary tumour was significantly lower than that of patients with ANX2-negative primary tumour \( (P = 0.0036) \).
DISCUSSION

In this study, we investigated the ANX2 expression in primary ccRCCs and their metastases. In agreement with earlier reports (Unwin et al, 2003; Domoto et al, 2007), ANX2 expression was upregulated in primary RCC compared with the corresponding normal renal cortex at mRNA and protein levels. Furthermore, western blot analysis showed that ANX2 protein level was more likely to be higher in primary tumours that developed metastases than in those that did not, as described in earlier report of rat RCC model (Tanaka et al, 2004). In immunohistochemical study, ANX2 was positive in 73 (47.4%) of the 154 primary ccRCCs and in 21 (87.5%) of the 24 metastatic tumours. The ANX2 positivity in primary tumour was clearly associated with advanced stage and a higher nuclear grade. Univariate and multivariate analysis showed that the immunohistochemical expression of ANX2 in a primary tumour is an independent predictor of metastasis.

The ANX2, a 34–36 kDa protein, is a multi-functional protein involved in cell proliferation and membrane physiology (Gerke et al, 2005). The dysregulation of ANX2 expression has been reported in various types of cancers, thus far. Upregulation of ANX2 expression has been reported in hepatocellular carcinoma (Frohlich et al, 1990), lung cancer (Cole et al, 1992), pancreatic cancer (Diaz et al, 2004; Esposito et al, 2006), breast cancer (Sharma et al, 2006), gastrointestinal cancer (Singh, 2007), glioma (Reeves et al, 1992) and colorectal cancer (Emoto et al, 2001b). Conversely, downregulation of ANX2 expression has been reported in prostate cancer (Chetchuti et al, 2001; Banerjee et al, 2003; Liu et al, 2003), osteosarcoma (Gillette et al, 2004), oesophageal squamous cell carcinoma (SCC) (Zhi et al, 2003) and head and neck SCC (Pena-Alonso et al, 2008). This discrepancy may be explained by ANX2 expression in the histological origin of tumours, the cellular localisation and the putative function of ANX2 in tumour cells. The ANX2 exists as a monomer in the cell cytoplasm or as a heterotetramer complexed with S100A10 in association with the plasma membrane. The ANX2 heterotetramer acts as a receptor for plasminogen, tissue-type plasminogen activator and tenascin C, and is implicated in extracellular matrix degradation (Gerke et al, 2005). The ANX2 heterotetramer acts as a receptor for plasminogen, tissue-type plasminogen activator and tenasin C, and is implicated in extracellular matrix degradation (Gerke et al, 2005). The ANX2 heterotetramer acts as a receptor for plasminogen, tissue-type plasminogen activator and tenasin C, and is implicated in extracellular matrix degradation (Gerke et al, 2005). The ANX2 heterotetramer acts as a receptor for plasminogen, tissue-type plasminogen activator and tenasin C, and is implicated in extracellular matrix degradation (Gerke et al, 2005). The ANX2 heterotetramer acts as a receptor for plasminogen, tissue-type plasminogen activator and tenasin C, and is implicated in extracellular matrix degradation (Gerke et al, 2005).

### Table 2 Univariate and multivariate analysis of metastasis-free and cancer-specific survival rate

|                        | Metastasis free (%) | Cancer-specific survival (%) |
|------------------------|---------------------|-----------------------------|
|                        | 5 years | 10 years | Log-rank test | Likelihood Ratio | Chi-square | Cox |
| **TNM stage**          |          |          |               |                |            |     |
| 1                      | 89.6    | 81.7     | 0.0003        | 3.2923         | 0.1928     |     |
| 2                      | 75.0    | 42.9     |               |                |            |     |
| 3                      | 52.5    | 46.7     |               |                |            |     |
| **Nuclear grade**      |          |          |               |                |            |     |
| 1                      | 88.5    | 82.9     | 0.0001        | 3.9556         | 0.1384     |     |
| 2                      | 79.5    | 68.0     |               |                |            |     |
| 3 or 4                 | 43.3    | 34.7     |               |                |            |     |
| **Microscopic venous invasion** |       |          |               |                |            |     |
| Negative               | 81.8    | 73.1     |              |                |            |     |
| Positive               | 74.1    | 60.2     |              |                |            |     |
| **Annexin II expression** |       |          |               |                |            |     |
| Negative               | 90.1    | 83.6     | <0.0001       | 5.3228         | 0.021      |     |
| Positive               | 63.0    | 46.5     |               |                |            |     |

#### Cox Proportional Hazards Regression

|                | Metastasis free (%) | Cancer-specific survival (%) |
|----------------|---------------------|-----------------------------|
|                | 5 years | 10 years | Log-rank test | Likelihood Ratio | Chi-square | Cox |
| **TNM stage** |          |          |               |                |            |     |
| 1              | 94.4    | 90.4     | <0.0001       | 26.7984        | <0.0001    |     |
| 2              | 91.8    | 73.3     |               |                |            |     |
| 3              | 74.1    | 63.6     |               |                |            |     |
| 4              | 25.2    | 0.0      |               |                |            |     |
| **Nuclear grade** |        |          |               |                |            |     |
| 1              | 97.6    | 97.6     | <0.0001       | 17.0858        | 0.0002     |     |
| 2              | 81.3    | 69.0     |               |                |            |     |
| 3 or 4        | 42.0    | 33.7     |               |                |            |     |
| **Microscopic venous invasion** |       |          |               |                |            |     |
| Negative      | 83.4    | 77.6     | 0.0011        | 0.0748         | 0.7845     |     |
| Positive      | 63.4    | 53.9     |               |                |            |     |
| **Annexin II expression** |       |          |               |                |            |     |
| Negative      | 84.2    | 81.9     | 0.0025        | 0.1022         | 0.7492     |     |
| Positive      | 66.5    | 54.8     |               |                |            |     |
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