patient ascites fluid for 14 days. The concentration of zoledronate was estimated by the expansion of Vγ9Vδ2 T cells. While zoledronate was not detectable in the ascites harvested after i.v. zoledornate injection, the zoledronate concentration in the ascites peaked 34.5 ± 20 nmol/L at 2 h, and rapidly declined to 10 nmol/L within 4 h after i.p. injection. The peak concentration reached higher and zoledronate concentration in the ascites sustained longer when zoledronate was i.p. injected than i.v. injected. These results indicated that local administration of zoledronate is important to sensitize tumor cells to Vγ9Vδ2 T-cell recognition.

The cytotoxicity of Vγ9Vδ2 T cells

Because tumor cells from patients 2319 and 2334 were negative for EpCAM, it was difficult to calculate their precise tumor load. In the other five patients, the number of $EpCAM^+$ tumor cells in ascites fluid were significantly reduced after zoledronate and Vγ9Vδ2 T-cell treatment (Fig. 3C). Immunoflueorscence microscopy revealed that Vγ9Vδ2 T cells attached to and surrounded $EpCAM^+$ tumor cells (Fig. 4A). These results are consistent with the cytological data (Fig. 4B). Large tumor cells and many leukocytes were present in the ascites. After Vγ9Vδ2 T-cell injection, a large number of small mononuclear lymphocytes, presumably the Vγ9Vδ2 T cells themselves, were observed in ascites. The number of large tumor cells was gradually reduced by the repetitive injections of Vγ9Vδ2 T cells. In addition, many polymorphonuclear leukocytes were recruited into the ascites after zoledronate injection. The cytotoxic activity of Vγ9Vδ2 T cells was also examined in vitro (Fig. 4D). When Vγ9Vδ2 T cells from patient 2325 were cocultured with autologous $EpCAM^+$ tumor cells in vitro, Vγ9Vδ2 T cells attached and killed tumor cells (movie clip S1). These results indicated that Vγ9Vδ2 T cells indeed recognized tumor cells and exert antitumor activity.

Clinical outcome

Clinical outcomes are summarized in Table 3. Patients 2305 and 2336 were withdrawn from the study after a single round of injections, due to disease progression. Patients 2307 and 2334 were withdrawn after one and three doses of Vγ9Vδ2 T cells due to aspiration pneumonia and bacterial infection of the central venous catheter, respectively (although both patients experienced relief of their clinical symptoms and showed promising signs of immunological reactivity reflected by induction of IFN-γ and the reduction of tumor cells in ascites). As shown in Figure 4C, bloody ascites of patient 2325 became clear after the treatment. In addition, the massive retention of ascites was no longer present (Fig. 5A). Ascites was also reduced and almost disappeared in patient 2328 (Fig. 5B); therefore he received an additional two rounds of injections. Excellent palliation of symptoms was observed in these patients. However, the clinical benefits of i.p. Vγ9Vδ2 T-cell injection were restricted to the local control of malignant ascites. Patients 2325 and 2328 developed mediastinal lymph node metastasis and bone metastasis, respectively.

Adverse events

None of the patients experienced abdominal pain or any other toxicity related to i.p. injection of Vγ9Vδ2 T cells. The most commonly observed treatment-related adverse events were fever (Grade 2: $n = 3$) and zoledronate-induced hypocalcemia (Grade 3: $n = 4$) (Tables 3 and S1). These events were generally mild-to-moderate in intensity and reversible. In contrast, most adverse events and symptoms were due to end-stage gastric cancer with peritoneal dissemination and disease progression, namely, loss of protein (Grade 2: $n = 1$, and Grade 3: $n = 5$) and electrolyte disorders (Grade 3: $n = 3$, and Grade 4: $n = 2$). Peritoneal dissemination caused serious complications, including intestinal obstruction and massive ascites, associated with weight loss, bloating, constipation, nausea, insomnia, and abdominal pain. Aspiration pneumonia (Grade 3) and disseminated intravascular coagulation (Grade 2) was observed in patients 2305 and 2319, respectively. Central venous catheter infection was detected in patients 2325 and 2334 (Grade 2). None of these adverse events was directly related to the administration of Vγ9Vδ2 T cells and there were no treatment-related deaths.

Discussion

We report here the direct evidence that adoptively transferred Vγ9Vδ2 T cells do indeed recognize tumor cells and exert antitumor effector activity in vivo. Previously, we had conducted a clinical trial of adoptive Vγ9Vδ2 T-cell transfer therapy for non-small cell lung cancer in patients who were refractory to other treatments [12, 13]. Autologous Vγ9Vδ2 T-cells were expanded ex vivo using zoledronate and IL-2, and administered six times at 2-week intervals. The cultured cells were well-tolerated and some clinical benefit was observed in some patients in whom Vγ9Vδ2 T cells were able to survive and expand [12, 13]. However, it remained to be determined whether transferred Vγ9Vδ2 T cells infiltrated into the tumor and exerted antitumor effector functions in vivo. Therefore, we conducted a trial of adoptive Vγ9Vδ2 T-cell therapy for patients with malignant ascites caused by advanced

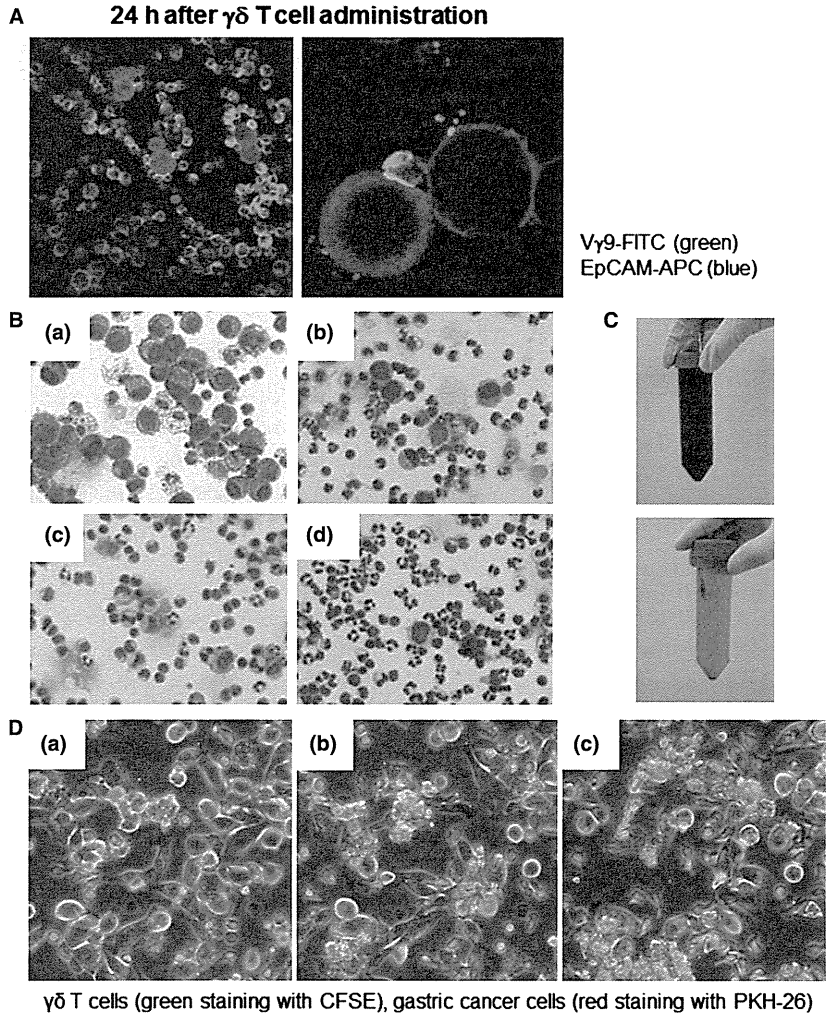

Figure 4. The cellular components and appearance of the ascites fluid. (A) The ascites fluid was harvested 24 h after Vγ9Vδ2 T-cell injection; the cells were stained with anti-TCRVγ9-FITC and anti-EpCAM-APC mAbs and examined by confocal fluorescence microscopy. The $EpCAM^+$ tumor cells (blue) are attached to and surrounded by Vγ9Vδ2 T cells (green) in ascites after Vγ9Vδ2 T-cell injections. Magnification was 150× on the left and 600× on the right. (B) Smears were prepared, air-dried, and stained with Diff-Quik (Sysmex, Kobe, Japan) according to the manufacturer's instructions. Cell morphology was evaluated using bright field microscopy (OLYMPUS BX41 with Canon EOS Kiss X4 digital camera, OLYMPUS, Tokyo, Japan, magnification 200×). Data from patient 2328 on day 0 (a: before zoledronate i.v.), day 9 (b: 24 h after 2nd Vγ9Vδ2 T-cell injection), day 21 (c: before zoledronate i.p.), and day 22 (d: 4 h after Vγ9Vδ2 T-cell injection) are shown. (C) The appearance of ascites from patient 2325 before and after four courses of Vγ9Vδ2 T-cell injections. (D) γδ T cells from patient 2325 (green staining with CFSE) recognized and killed autologous $EpCAM^+$ gastric cancer cells purified from ascites fluid (red staining with PKH-26), by direct contact. Tumor cells were attacked by the γδ T cells; collapse of the cell membranes led to apoptosis. It took approximately 2 h to progress from (a) to (c). Movie clip S1 is also provided.

gastric cancer. PBMC were harvested by apheresis; Vγ9Vδ2 T cells were similarly prepared with zoledronate and IL-2; Vγ9Vδ2 T cells were injected weekly into the peritoneal cavity, four times in total (Fig. 1B). Direct injection of Vγ9Vδ2 T cells into the peritoneal cavity allows them direct access to the tumor cells, bypassing the difficulties of recruitment of transferred Vγ9Vδ2 T-cells into solid tumors.

As shown in Figure 4A, many Vγ9Vδ2 T cells attached to each $EpCAM^+$ tumor cell in the ascites 24 h after their i.p. injection. Concomitantly, IFN-γ was detected in ascites with kinetics similar to the increased number of Vγ9Vδ2 T cells (Fig. 3B). The number of tumor cells in ascites was significantly reduced even after the first cell transfer and remained substantially lower during the course of the treatment (Fig. 3C). These results document tumor cell recognition and antitumor activity of Vγ9Vδ2 T cells in vivo. When autologous tumor cells were isolated by anti-EpCAM magnetic beads and cocultured with autologous zoledronate-expanded Vγ9Vδ2 T cells,

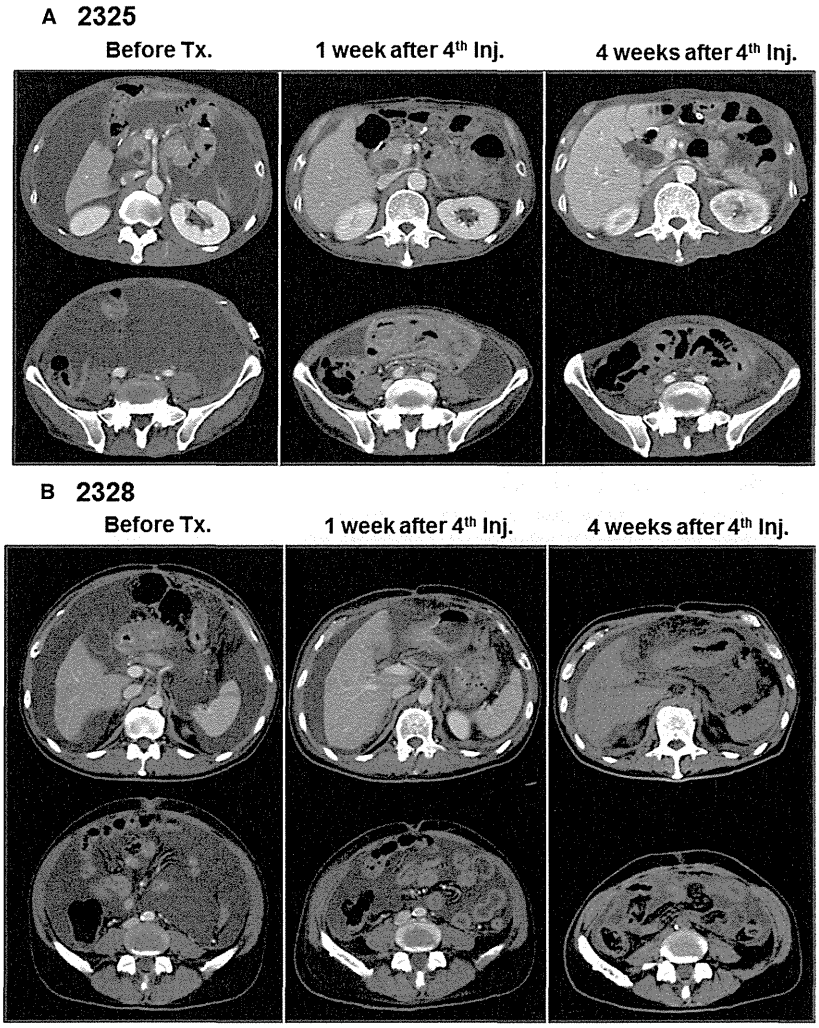

Figure 5. Computed tomography findings in patients 2325 (A) and 2328 (B). Retention of a large amount of ascites was observed before treatment (left panels). The amount of ascites was reduced 1 week (middle panels) and 4 weeks (right panels) after four courses of Vγ9Vδ2 T-cell injections.

Vγ9Vδ2 T cells indeed recognized and killed autologous tumor cells (Fig. 4D and movie clip S1). Such antitumor activity of i.p. Vγ9Vδ2 T cells resulted in some remarkable clinical effects. While the appearance of ascites was initially bloody in patient 2325, it became clear after i.p. Vγ9Vδ2 T-cell treatment (Fig. 4C). The reduction in ascites fluid was confirmed by computed tomography in patients 2325 and 2328 (Fig. 5). These results indicate that i.p. Vγ9Vδ2 T-cell injection combined with zoledronate contributed to the local control of malignant ascites in some patients with gastric cancer for whom no standard therapy apart from paracentesis was available.

NBPs such as zoledronate are widely used in the clinic for the treatment of bone metastases and are known as potent stimulators of Vγ9Vδ2 T cells [32]. Zoledronate blocks the mevalonate pathway, leading to intracellular accumulation of IPP, its isomer dimethylallyl pyrophosphate (DMAPP) and ApppI [24, 33, 34]. Because Vγ9Vδ2 T cells recognize these mevalonate metabolites in tumor cells, the high amounts of IPP and ApppI in zoledronate-treated tumor cells contributes to their recognition and lysis [35]. In the present study, zoledronate was administered 24 h prior to i.p. Vγ9Vδ2 T-cell injection with the aim of presensitizing the tumor cells. We injected zoledronate either i.v. or i.p. and compared these routes of injection (Fig. 1B). As shown in Figure 3B, smaller amounts of IFN-γ in ascites were detected in two of six patients after i.v. zoledronate injection, while higher amounts were found in ascites of all four patients who received i.p. zoledronate. These results are consistent with pharmacokinetic data for zoledronate, indicating that serum concentrations decline rapidly after infusion [31].

Table 3. Clinical outcome

Patient ID	Numbers of γδ T-cell injection	Adverse events (Grade, CTCAE v. 4.0)	Clinical outcome	
			Ascites	Others
2305	1	Rectal obstruction (3)[1]	No change	Growth of primary lesion
2307	1	Aspiration (3)[1], nausea (3),tumor pain (3), insomnia (2), hypoalbuminemia (2)	No change	Pleural effusion
2319	4	Fatigue (3), weight loss (3), hyponatremia (4), hypocalcemia (3), hypoalbuminemia (3), hypophosphatemia (3), female genital tract fistula (1), urinary tract infection (3), depressed level of consciousness (3), disseminated intravascular coagulation (2), lymphocyte count decreased (3)	No change	Obstructive jaundice due to the growth of primary lesion
2325	4	Fever (2), bloating (2), constipation (2), nausea (2), anemia (1), hypoalbuminemia (3), hypophosphatemia (3), hypocalcemia (3), urinary tract infection (2), insomnia (2), tumor pain (3), central venous catheter-related infection (2)	Disappeared	Mediastinal lymphadenopathy, pleural effusion, carcinomatous lymphangiosis
2334	3	Fever (2), nausea (2), insomnia (2), central venous catheter-related infection (2)[1], tumor pain (3), anemia (3), hypocalcemia (3), hypoalbuminemia (3), palmar-plantar erythrodysesthesia syndrome (1)	No change	Metastasis to ovary
2336	1	Tumor pain (3), hypoalbuminemia (3), hypocalcemia (3), hyponatremia (3), hyperkalemia (4)	No change	Poor performance status[1], metastasis to bladder, ovary and skin
2328	4 (+2)	Fever (2), gastritis (2), constipation (2), hypoalbuminemia (3), lymphocyte count decreased (3)	Reduced	Bone metastasis

[1]Cause for discontinuance.

When we harvest ascites 2–8 h after i.p. zoledronate injection, ascites fluid contained the sufficient amount of zoledronate to expand Vγ9Vδ2 T cell, suggesting they might inhibit farnesyl pyrophosphate (FPP) synthase activity in the tumor cells at this time point (Fig. S1). However, Vγ9Vδ2 T cell did not respond to the ascites fluid harvested after i.v. zoledronate injection. Therefore, the zoledronate concentration in the ascites might not be sufficient for the inhibition of FPP synthase activity after i.v. administration. While the optimum dose and timing of zoledronate administration remain to be elucidated, the local administration of zoledronate is desired to inhibit FPP synthase and sensitize tumor cells in the abdominal cavity to efficient Vγ9Vδ2 T-cells recognition.

In addition to the direct cytotoxic activity of Vγ9Vδ2 T-cells on the tumor cells, their activation results in release of many cytokines and chemokines that may lead to the recruitment and activation of other immune cells. It has been reported that Vγ9Vδ2 T cells induce dendritic cell maturation [36], B-cell activation [37], and polarization of Th1 immune responses [38]. We observed marked recruitment of neutrophils into the peritoneal cavity in this study (Fig. 4B); zoledronate alone induced granulocyte recruitment, suggesting that NBPs induce γδ T cell-independent neutrophil recruitment in humans. Recently, Norton et al. [39] reported that intraperitoneal injection of alendronate, one of the FDA-approved NBPs, induced peritoneal inflammation in mice. In their model, neutrophil recruitment depended on mast cells and IL-1R signaling. As mice lack the counterpart of human Vγ9Vδ2 T cells and thus cannot respond to IPP and NBPs, the mechanism of peritoneal inflammation in mice might be different from our human study. Consistent with a previous reports that Vγ9Vδ2 T-cell activation-induced neutrophil migration and increased their phagocytic potential and release of α-defensins [40], and that γδ T cells rapidly induce CXCL8-mediated migration of neutrophils [41], infiltration of neutrophils was sustained after i.p. Vγ9Vδ2 T-cell injection in this study (Fig. 4B). Despite the recruitment of many neutrophils into the peritoneal cavity, patients did not complain of abdominal pain and did not display any signs of peritonitis except retention of ascites after Vγ9Vδ2 T-cell injection.

The combination of i.p. Vγ9Vδ2 T-cell injection and zoledronate for the treatment of malignant ascites had acceptable tolerability without unexpected severe or long-lasting adverse events. Because patients with severe peritoneal dissemination and malignant ascites are generally in a poor condition, adverse events were frequent; many of them were not associated with cell transfer (Tables 3 and S1). However, pyrexia was probably associated with the release of proinflammatory cytokines induced by zoledronate and Vγ9Vδ2 T-cell injection. The local i.p. injection of zoledronate and Vγ9Vδ2 T cells might reduce the

systemic adverse events associated with the release of pro-inflammatory cytokines. Though the kinetics of IL-1β, IL-8, and TNF-α production in ascites fluid were similar with that of IFN-γ, the changes of these cytokines were not detected in the patients' serum (data not shown). The IL-6 was elevated before the treatment in many of these advanced cancer patients, the changes associated with zoledronate and/or Vγ9Vδ2 T-cells were not clear. The alterations in laboratory parameters were rarely considered clinically relevant to the treatment except for hypocalcemia caused by zoledronate.

The patients in this study received S-1 plus cisplatin, S-1 plus docetaxel, or docetaxel alone as a standard regimen for the treatment of unresectable or recurrent gastric cancer prior to the Vγ9Vδ2 T-cell therapy (Table 1) [17, 18]. It has been reported that the overall median survival time in treatment-naïve patients with malignant ascites was approximately 5 months irrespective of the regimen received [16, 42, 43]. Once patients have become refractory to these chemotherapies, it is unlikely that they will experience a survival benefit from any treatment. In such cases, paracentesis and diuretics are primarily used in managing malignant ascites, neither of which is an anti-cancer treatment but solely palliative [15]. In contrast, the i.p. injection of Vγ9Vδ2 T cells combined with zoledronate directly affects the tumor cells and reduces their number in the peritoneal cavity, as well as decreasing the amount of ascites fluid, leading to palliation of the symptoms of malignant ascites.

Although the i.p. Vγ9Vδ2 T-cell injection and zoledronate treatment is unlikely to impact overall survival in such advanced disease, especially with metastasis, our results show a clear clinical benefit for the local control of malignant ascites (Fig. 5). We are planning to conduct a new clinical trial for treatment-naïve patients with peritoneal dissemination to evaluate the survival benefit of this treatment. Furthermore, combinations of this newly emerging therapy with established surgical, radiotherapy, and chemotherapy treatments are expected to improve the survival of cancer patients in future.

Acknowledgements

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Conflict of Interest

Dr. Kazuhiro Kakimi received research support from Medinet Co. Ltd. (Yokohama, Japan). The costs of the entire γδ T cell culture production and part of the immunological assays were covered by Medinet Co. Ltd. The study sponsors had no involvement in study design; collection, analysis, and interpretation of data; writing the report; and the decision to submit the report for publication. All other authors have declared there are no financial conflicts of interest related to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The concentration of zoledronate was estimated by the Vγ9Vδ2 T-cell bioassay. PBMCs from healthy donor were stimulated with indicated amount of zoledronate in AlyS203 medium containing 1000 IU/mL human recombinant IL-2 and 10% pooled human serum. After 14 day-culture, expansion of Vγ9Vδ2 T cell was measured by flow cytometry to prepare the standard curve. Same donor-derived PBMCs were cultured in IL-2 containing medium and in the presence of 10% patient ascites fluid for 14 days. The concentration of zoledronate was estimated by the expansion of Vγ9Vδ2 T cell using the standard curve.
Movie clip S1. Patient's Vγ9Vδ2 T cells recognize and kill autologous tumor cells.
Table S1. Adverse events.
Table S2. % Cytotoxicity of Vγ9Vδ2 T cells against Daudi cells w/o zoledronate treatment.

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ORIGINAL ARTICLE – UROLOGIC ONCOLOGY

Prognostic Significance of CD204-Positive Macrophages in Upper Urinary Tract Cancer

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ABSTRACT

Background. Evidence suggests that CD204-positive ($CD204^+$) tumor-infiltrating macrophages are associated with aggressive behavior of various cancers; however, the clinical, pathological, and prognostic associations of tumor-infiltrating $CD204^+$ macrophages in urothelial cancer have not been reported.

Methods. A tissue microarray was constructed from the centers and peripheries of 171 upper urinary tract cancers treated with nephroureterectomy. CD204 immunohistochemistry was performed. The density of $CD204^+$ cells was calculated using image analysis software, and survival analyses were performed using the Kaplan–Meier method and multivariate Cox proportional hazards regression models.

Results. High $CD204^+$ cell density at the centers and peripheries of tumors was significantly associated with several adverse prognostic factors, including sessile architecture, histological high-grade, presence of lymphovascular invasion, concomitant carcinoma in situ, higher tumor stage, and lymph node metastasis. High $CD204^+$ cell density was significantly associated with shorter metastasis-free and cancer-specific survival (log-rank $p < 0.001$) and shorter metastasis-free survival in multivariate analysis.

Conclusions. A high density of tumor-infiltrating $CD204^+$ macrophages was associated with aggressive behavior of upper urinary tract cancer. Our results suggest that a specific immune microenvironment may be associated with the biological behavior of urothelial cancer and that CD204 may serve as a novel prognostic biomarker for these tumors.

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Tumor tissue comprises variable numbers of cancer and stromal cells, and the tumor microenvironment plays important roles in the biological behavior of cancer.[1–6] Macrophages, which contribute to the host's immune response, are the most abundant stromal cells in tumors.[7] Macrophages possess tumor suppressive (M1) and tumor-supportive (M2) functions.[8] M2-polarized macrophages express high levels of CD204 (also called scavenger receptor A).[9] Recent studies show that a high number of tumor-infiltrating CD204-positive ($CD204^+$) macrophages is associated with worse patient outcome in a variety of cancers.[10–16] However, the role of $CD204^+$ macrophages in urothelial cancer has not been reported. We therefore examined the clinicopathological and prognostic associations of tumor-infiltrating $CD204^+$ macrophages in patients with upper urinary tract cancer.

MATERIALS AND METHODS

Study Population

A total of 171 patients with upper urinary tract cancer who underwent nephroureterectomy at The University of Tokyo Hospital from 1996 to 2012 were included in this study. No

patient received neoadjuvant chemotherapy. In 31 cases, bladder cancer was found and treated before or at the time of nephroureterectomy. All research protocols in the present study were approved by our Institutional Review Board.

Histopathological Evaluation

Hematoxylin and eosin (H&E)-stained slides of all cases were reviewed by a pathologist (TM) without knowledge of clinical outcomes. Tumor histology and grade were defined according to the World Health Organization/International Society of Urologic Pathology consensus classification.[17] All tumors (103 pelvic cancers and 68 ureteral cancers) were histologically diagnosed as urothelial carcinomas. Tumors were staged according to the TNM classification system.[18] Lymphovascular invasion was examined using H&E and Elastica van Gieson staining.

Tissue Microarray Construction

H&E-stained slides were evaluated for the presence of the tumor center and the periphery. The tumor periphery was defined as the area of invasive margin or as the area where cancer cells were closest to the lamina propria for non-invasive tumors. For each tumor specimen, H&E-stained slides containing the tumor center and the periphery were selected. Using a tissue microarrayer (Beecher Instruments Inc., Sun Prairie, WI, USA), each region in the donor paraffin block was cored with a needle 2 mm in diameter and transferred to the recipient paraffin block.[19]

Immunohistochemical Analysis

Preparation of sections from paraffin blocks was performed as previously described.[20] Immunohistochemical analysis of CD204 was performed using a mouse monoclonal antibody against human CD204 (clone SRA-E5, 1:500; Transgenic, Kumamoto, Japan) according to standard techniques for a Ventana Benchmark® XT Autostainer (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was carried out using Cell Conditioning Solution (CC1-Tris-based EDTA buffer, pH 8.0; Ventana Medical Systems). Visualization was achieved using the I-VIEW DAB Universal Kit (Ventana Medical Systems) and hematoxylin counterstaining.

Image Analysis

Images of immunostained slides were digitized at 20× magnification using the NanoZoomer Digital Pathology (NDP) System (Hamamatsu Photonics, Hamamatsu, Japan). For digital quantification, image analysis software (Tissue Studio v.3.5; Definiens AG, Munich, Germany) was used to distinguish the $CD204^+$ macrophages.[21] The percentage of the area containing $CD204^+$ cells (summed area with $CD204^+$ cells/total measured area × 100) was calculated for each tissue microarray core.[14]

Statistical Analysis

All statistical analyses were performed using SAS software (Version 9.3, SAS Institute, Cary, NC, USA). All p values were two-sided. Differences were considered significant at $p < 0.05$. For categorical data, the Chi square test was performed. The Kaplan–Meier method and log-rank test were used to analyze survival. To control for confounding variables, multivariate Cox proportional hazards regression models were used. The multivariate models initially included gender, age at diagnosis, tumor side, tumor location, tumor architecture, tumor grade, lymphovascular invasion, concomitant carcinoma in situ, tumor stage, lymph node metastasis, and adjuvant chemotherapy. Tumor stage was dichotomized (pTa–pT1 vs. pT2–pT4) to be consistent with previous studies.[22–26] A backward elimination was performed using a threshold of $p = 0.05$; however, CD204 status, tumor stage, and lymph node metastasis were forced into the final models.

RESULTS

Distribution of Tumor-Infiltrating $CD204^+$ Macrophages in Upper Urinary Tract Cancer

Representative photomicrographs of CD204 immunohistochemistry are presented in Fig. 1. The median tumor-infiltrating $CD204^+$ cell density was 0.64 % (range 0.03–9.29 %) at the tumor center, and 0.81 % (range 0.03–12.43 %) at the tumor periphery. $CD204^+$ cell density of the two areas correlated positively (Spearman $r = 0.76$; $p < 0.0001$), and there was no significant difference between the tumor center and the periphery ($p > 0.05$, Wilcoxon signed-rank test). We divided the cases into high and low $CD204^+$ groups according to the median value of $CD204^+$ cell density.[14] High $CD204^+$ density at both the tumor center and the periphery was significantly associated with sessile architecture, histological high-grade, presence of lymphovascular invasion, concomitant carcinoma in situ, higher tumor stage, and lymph node metastasis (Table 1).

$CD204^+$ Macrophages and Clinical Outcome of Upper Urinary Tract Cancer

Patients with previous or concurrent bladder cancer ($n = 31$) were excluded from the survival analyses because

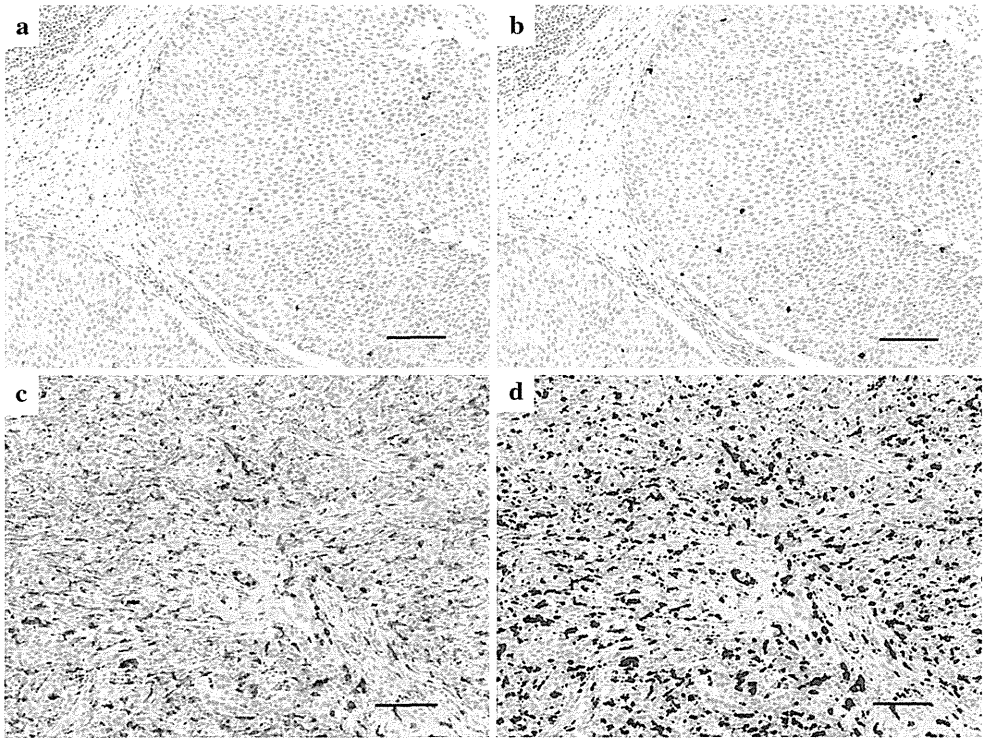

FIG. 1 Quantitation of $CD204^+$ macrophage density in upper urinary tract urothelial carcinoma. CD204 immunohistochemistry shows low (**a**) and high (**c**) infiltration of $CD204^+$ macrophages. Using image analysis software, immunopositive areas are highlighted in red, and the percentage of immunopositive area (summed area with $CD204^+$ macrophages/total measured area × 100) was calculated (**b**, **d**). *Bars* indicate 100 μm. *$CD204^+$* CD204-positive

they may affect the outcome of upper urinary tract cancer.[27] Among the 140 patients without bladder cancer at the time of nephroureterectomy, there were 58 bladder recurrences, 32 metastases, and 23 cancer-specific deaths during a median 56-month follow-up (interquartile range 25–86 months).

Kaplan–Meier analysis revealed that high $CD204^+$ density at both the tumor center and the periphery was significantly associated with shorter metastasis-free and cancer-specific survival (log-rank $p < 0.001$; Fig. 2). High $CD204^+$ density at both the tumor center and the periphery was also significantly associated with shorter metastasis-free survival in univariate and multivariate Cox models (Table 2). High $CD204^+$ density was significantly associated with shorter cancer-specific survival in univariate analysis, but the statistical significance was not achieved in multivariate analysis (Table 3). Lymphovascular invasion and lymph node metastasis were also significantly associated with shorter metastasis-free and cancer-specific survival in univariate and multivariate analyses. Tumor stage was not significantly associated with shorter metastasis-free and cancer-specific survival in multivariate analysis, consistent with previous studies.[24–26,28] $CD204^+$ density was not significantly associated with bladder recurrence-free survival in Kaplan–Meier analysis (Fig. 2) or in univariate and multivariate Cox models (Supplementary Table 1).

DISCUSSION

To our knowledge, the present study is the first to assess the clinicopathological and prognostic associations of $CD204^+$ macrophages in upper urinary tract cancer. We found that a high density of tumor-infiltrating $CD204^+$ cells was significantly associated with sessile architecture, histological high-grade, lymphovascular invasion, concomitant carcinoma in situ, higher tumor stage, and lymph node metastasis, which are adverse prognostic factors in upper urinary tract cancer.[29,30] Moreover, high $CD204^+$ cell density was significantly associated with shorter metastasis-free and cancer-specific survival. Our findings suggest that tumor-infiltrating $CD204^+$ macrophages are associated with aggressive behavior of upper urinary tract cancer.

A high density of tumor-infiltrating $CD204^+$ macrophages is associated with a poorer prognosis in lung,[10,12,13] pancreatic,[11,14,15] and esophageal carcinomas.[16] In two of these studies, the independent prognostic significance of CD204 expression was also shown using multivariate analysis.[12,14] In contrast, there was no prognostic

TABLE 1 Correlation between tumor-infiltrating CD204-positive macrophage density and clinicopathological features in patients with upper urinary tract cancer who underwent nephroureterectomy

Clinical or pathologic feature	Total *N*	CD204 (tumor center) [*n* (%)]		*p* value	CD204 (tumor periphery) [*n* (%)]		*p* value
		Low	High		Low	High	
All cases	171	85 (50)	86 (50)		85 (50)	86 (50)	
Gender				0.34			0.96
Men	119	62 (52)	57 (48)		59 (50)	60 (50)	
Women	52	23 (44)	29 (56)		26 (50)	26 (50)	
Age (years)				0.14			0.59
<70	93	51 (55)	42 (45)		48 (52)	45 (48)	
≥70	78	34 (44)	44 (56)		37 (47)	41 (53)	
Side				0.25			0.82
Left	86	39 (45)	47 (55)		42 (49)	44 (51)	
Right	85	46 (54)	39 (46)		43 (51)	42 (49)	
History of bladder cancer				0.81			0.81
No	140	69 (49)	71 (51)		69 (49)	71 (51)	
Yes	31	16 (52)	15 (48)		16 (52)	15 (48)	
Tumor location				0.03			0.23
Renal pelvis	103	58 (56)	45 (44)		55 (53)	48 (47)	
Ureter	68	27 (40)	41 (60)		30 (44)	38 (56)	
Tumor architecture				0.0037			0.0037
Papillary	126	71 (56)	55 (44)		71 (56)	55 (44)	
Sessile	45	14 (31)	31 (69)		14 (31)	31 (69)	
Grade				<0.0001			<0.0001
Low	19	18 (95)	1 (5.3)		19 (100)	0	
High	152	67 (44)	85 (56 %)		66 (43)	86 (57)	
Lymphovascular invasion				<0.0001			<0.0001
Absent	97	61 (63)	36 (37 %)		62 (64)	35 (36)	
Present	74	24 (32)	50 (68)		23 (31)	51 (69)	
Concomitant carcinoma in situ				0.0046			0.0002
Absent	88	53 (60)	35 (40)		56 (64)	32 (36)	
Present	83	32 (39)	51 (61)		29 (35)	54 (65)	
Tumor stage				<0.0001			<0.0001
pTa	37	34 (92)	3 (8.1)		35 (95)	2 (5.4)	
pTis	7	4 (57)	3 (43)		3 (43)	4 (57)	
pT1	31	17 (55)	14 (45)		21 (68)	10 (32)	
pT2	18	8 (44)	10 (56)		6 (33)	12 (67)	
pT3	69	22 (32)	47 (68)		19 (28)	50 (72)	
pT4	9	0	9 (100)		1 (11)	8 (89)	
Lymph node metastasis				0.0017			0.0081
Absent	152	82 (54)	70 (46)		81 (53)	71 (47)	
Present	19	3 (16)	16 (84)		4 (21)	15 (79)	

significance of $CD204^+$ macrophages in renal cell carcinoma[31] or lymphoma.[32] In the present study, univariate and multivariate analysis show that tumor-infiltrating $CD204^+$ cell density associated significantly with shorter metastatic-free survival. Statistical significance was not achieved for cancer-specific survival in multivariate analysis, probably owing to fewer events and lower statistical power. Our results suggest that tumor-infiltrating $CD204^+$ cell density may be a novel prognostic biomarker to predict metastasis in patients with upper urinary tract cancer.

To assess heterogeneity within a tumor, we evaluated two tissue cores taken from either the tumor center or the periphery for each case. While most previous studies on CD204 visually evaluated a few high-power

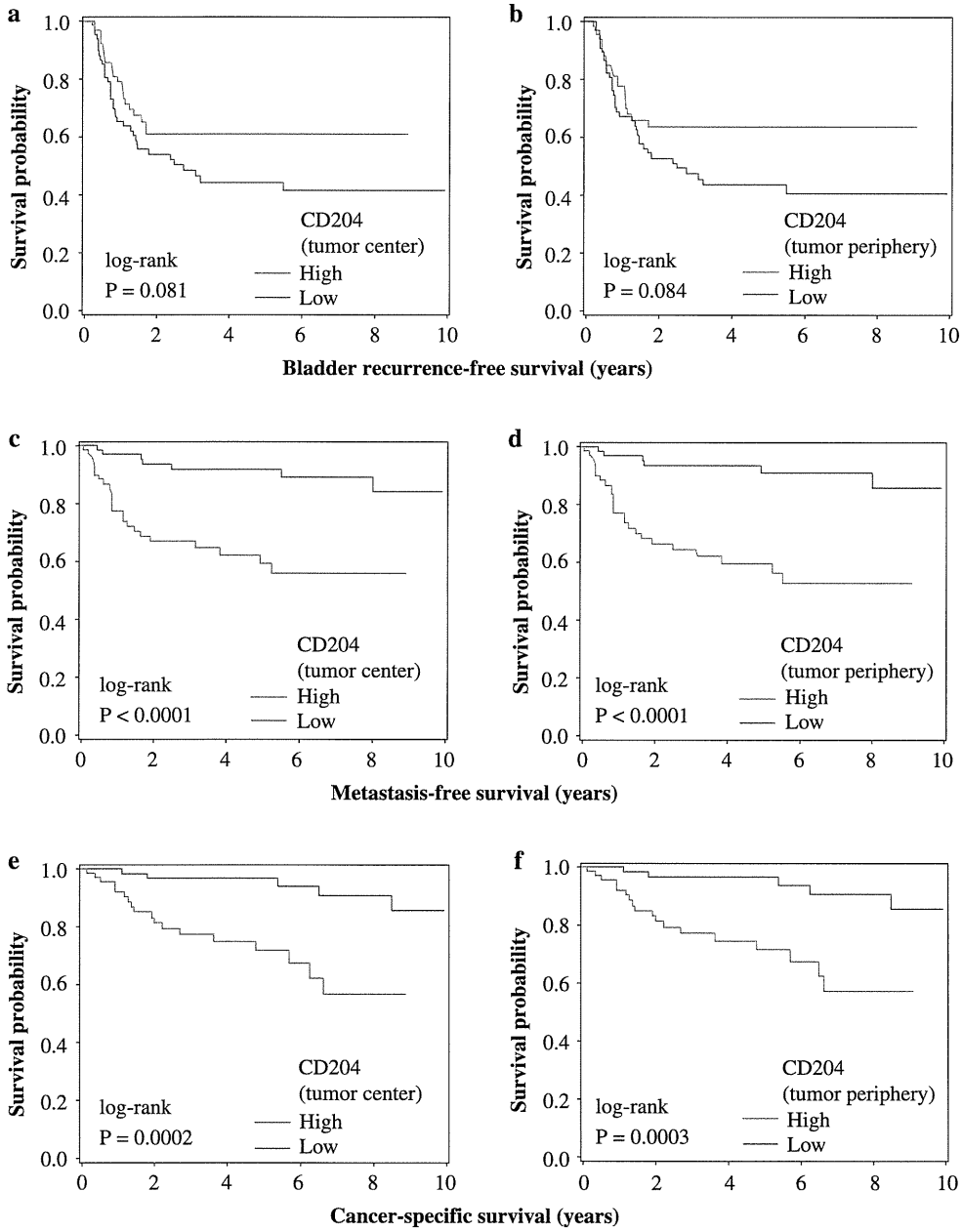

FIG. 2 Kaplan–Meier analysis of bladder recurrence-free survival (**a**, **b**), metastasis-free survival (**c** and **d**), and cancer-specific survival (**e**, **f**) after nephroureterectomy according to $CD204^+$ macrophage density at the tumor center (**a**, **e**) and periphery (**b**, **f**). *$CD204^+$* $CD204^+$-positive

fields,[10–13,16,32,33] here we evaluated $CD204^+$ cell density in a larger area (>6 mm^2 for each case) using validated image analysis software.[21] We found that $CD204^+$ cell density at the tumor center and the periphery strongly correlated, and that the associations of CD204 expression with clinicopathological features and patient outcomes were quite similar between them. Although further studies using whole slide sections are required to validate our findings,[34,35] our results suggest that $CD204^+$ cell density at either the tumor center or periphery may serve as a useful prognostic marker for upper urinary tract cancer.

Accumulating evidence suggests that tumor cells induce tumor-promoting $CD204^+$ macrophages to generate a specific microenvironment that supports tumor progression. Tumor-associated macrophages, which are recruited to tumors by multiple growth factors and chemokines that are often produced by tumor cells,[8,36] induce the proliferation, survival, and invasion of tumor cells by producing a wide range of factors, including matrix metalloproteinases and growth factors such as fibroblast growth factor and epidermal growth factor.[37–39] A recent study by Neyen et al.[40] shows that tumor progression and metastasis are inhibited

TABLE 2 Tumor-infiltrating CD204-positive macrophage density in upper urinary tract cancer and patient outcomes (metastasis)

	Univariate analysis		Multivariate analysis			
	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value
CD204 (tumor center) (high vs. low)	4.78 (2.06–11.1)	0.0003	2.52 (1.02–6.22)	0.045	–	–
CD204 (tumor periphery) (high vs. low)	5.86 (2.40–14.3)	0.0001	–	–	3.10 (1.17–8.16)	0.022
Sex (female vs. male)	1.00 (0.46–2.17)	0.99	–	–	–	–
Age (≥70 vs. <70 years)	2.03 (1.00–4.12)	0.050	–	–	–	–
Side (right vs. left)	0.86 (0.43–1.72)	0.66	–	–	–	–
Tumor location (ureter vs. renal pelvis)	1.90 (0.95–3.80)	0.071	–	–	–	–
Tumor architecture (sessile vs. papillary)	1.57 (0.76–3.26)	0.23	–	–	–	–
Tumor grade (high vs. low)[a]	–	–	–	–	–	–
Lymphovascular invasion (present vs. absent)	6.73 (2.90–15.6)	<0.0001	2.79 (1.02–7.65)	0.046	3.03 (1.08–8.49)	0.035
Concomitant carcinoma in situ (present vs. absent)	3.16 (1.49–6.68)	0.0026	–	–	–	–
Tumor stage (pT2–pT4 vs. pTa–pT1)	9.84 (2.99–32.4)	0.0002	2.69 (0.64–11.4)	0.18	1.89 (0.41–8.80)	0.42
Lymph node metastasis (present vs. absent)	6.37 (3.13–12.9)	<0.0001	2.30 (1.07–4.94)	0.034	2.64 (1.25–5.55)	0.011
Adjuvant chemotherapy	2.87 (1.43–5.78)	0.0031	–	–	–	–

The multivariate Cox regression models initially included CD204 status (tumor center or periphery), gender, age at diagnosis, tumor side, tumor location, tumor architecture, tumor grade, lymphovascular invasion, concomitant carcinoma in situ, tumor stage, lymph node metastasis, and adjuvant chemotherapy. A backward elimination was performed with a threshold of $p = 0.05$; however, CD204 status, tumor stage, and lymph node metastasis were forced into the final models

CI confidence interval, *HR* hazard ratio

[a] Because patients with low-grade tumors did not experience an event, the hazard ratio could not be calculated

TABLE 3 Tumor-infiltrating CD204-positive macrophage density in upper urinary tract cancer and patient outcomes (cancer-specific mortality)

	Univariate analysis		Multivariate analysis			
	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value
CD204 (tumor center) (high vs. low)	5.41 (1.99–14.8)	0.001	2.75 (0.93–8.11)	0.067	–	–
CD204 (tumor periphery) (high vs. low)	5.19 (1.91–14.1)	0.001	–	–	2.50 (0.87–7.20)	0.089
Sex (female vs. male)	0.98 (0.39–2.50)	0.97	–	–	–	–
Age (≥70 vs. <70 years)	2.78 (1.19–6.52)	0.019	–	–	–	–
Side (right vs. left)	0.84 (0.37–1.91)	0.67	–	–	–	–
Tumor location (ureter vs. renal pelvis)	1.50 (0.66–3.44)	0.34	–	–	–	–
Tumor architecture (sessile vs. papillary)	2.15 (0.94–4.91)	0.069	–	–	–	–
Tumor grade (high vs. low)[a]	–	–	–	–	–	–
Lymphovascular invasion (present vs. absent)	13.4 (3.96–45.6)	<0.0001	5.15 (1.27–20.8)	0.022	5.24 (1.28–21.5)	0.022
Concomitant carcinoma in situ (present vs. absent)	3.30 (1.36–8.03)	0.0085	–	–	–	–
Tumor stage (pT2–pT4 vs. pTa–pT1)	22.7 (3.05–168.6)	0.0023	3.78 (0.38–37.7)	0.26	3.09 (0.28–33.7)	0.35
Lymph node metastasis (present vs. absent)	7.45 (3.26–17.0)	<0.0001	2.22 (0.92–5.35)	0.075	2.70 (1.15–6.32)	0.022
Adjuvant chemotherapy	3.63 (1.54–8.57)	0.0033	–	–	–	–

The multivariate Cox regression models initially included CD204 status (tumor center or periphery), gender, age at diagnosis, tumor side, tumor location, tumor architecture, tumor grade, lymphovascular invasion, concomitant carcinoma in situ, tumor stage, lymph node metastasis, and adjuvant chemotherapy. A backward elimination was performed with a threshold of $p = 0.05$; however, CD204 status, tumor stage, and lymph node metastasis were forced into the final models

CI confidence interval, *HR* hazard ratio

[a] Because patients with low-grade tumors did not experience an event, the hazard ratio could not be calculated

in CD204-knockout mice in two in vivo models of ovarian and pancreatic cancer. Moreover, treatment of tumor-bearing mice with 4F, a small peptide ligand of CD204 that competes with physiological CD204 ligands, inhibited tumor progression and metastasis.[40] Taken together, these observations suggest that tumor cells and $CD204^+$ macrophages may cooperate to contribute to more aggressive tumor behavior and that CD204 may be a potential drug

target in the prevention of metastatic cancer progression. Therefore, further studies on the crosstalk between urothelial cancer cells and $CD204^+$ macrophages are warranted.

Whereas $CD204^+$ cell density was associated with shorter metastasis-free and cancer-specific survival, it was not associated with bladder recurrence-free survival. It is likely that the ability of an upper urinary tract cancer to recur elsewhere in the urothelium may involve a different pathway. Whether other immune microenvironment markers such as lymphocyte surface antigens,[41–44] cytokines,[45,46] or chemokine receptors[45] can predict bladder recurrence is an important subject for future studies.

CONCLUSIONS

Tumor-infiltrating $CD204^+$ cell density significantly associated with adverse prognostic factors and shorter metastatic-free and cancer-specific survival in patients with upper urinary tract cancer. Although further studies are required to validate our findings, our results suggest that a specific immune microenvironment may be associated with biological behavior of urothelial cancer and that CD204 may serve as a novel prognostic biomarker for these tumors.

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CONFLICT OF INTEREST Takashi Ichimura, Teppei Morikawa, Taketo Kawai, Tohru Nakagawa, Hirokazu Matsushita, Kazuhiro Kakimi, Haruki Kume, Shumpei Ishikawa, Yukio Homma, and Masashi Fukayama declare no conflicts of interest.

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Original contribution

Prognostic impact of microRNA-145 down-regulation in adult T-cell leukemia/lymphoma☆,☆☆

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Summary Adult T-cell leukemia/lymphoma (ATL) is a highly aggressive tumor caused by human T-cell leukemia virus type 1. MicroRNAs (miRNAs) are closely involved in the development and progression of various tumors. Here we investigated the dysregulation of miRNAs in ATL and its clinical significance. Studies using miRNA arrays and subsequent real-time reverse transcription polymerase chain reaction showed that, in the 9 ATL cell lines examined, 1 miRNA was consistently up-regulated, whereas another 3 were consistently down-regulated, compared with normal CD4-positive lymphocytes. Next, we analyzed the prognostic impact of these 4 miRNAs in patients with aggressive-type ATL (n = 40). Of the 4 dysregulated miRNAs selected, 3 (miR-130b higher expression, miR-145 lower expression, and miR-223 lower expression) were significantly associated with a worsened overall patient survival. We found that expressions of these 3 miRNAs were correlated with each other. To clarify which of the 3 had the most significant impact on overall survival, we performed a multivariate prognostic analysis that included these 3 miRNAs, and only miR-145 lower expression was selected as an independent risk factor (P = .0005). When overexpressed in an ATL cell line in vitro, miR-145 specifically inhibited tumor cell growth. In conclusion, our study suggests that miR-145 down-regulation provides a growth advantage in ATL and is highly associated with a worsened prognosis for patients with ALT. Hence, miR-145 may be a useful prognostic marker and a potential therapeutic target for ATL.

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1. Introduction

MicroRNAs (miRNAs) are 18- to 25-nucleotide, single-stranded noncoding RNA molecules that play an important regulatory role by targeting mRNAs for repressing translation or mRNA cleavage [1]. They are transcribed with primary miRNAs, transported into the cytoplasm, cleaved into mature miRNAs, and then loaded onto the miRNA-induced silencing complex for RNA interference. The miRNA-induced silencing complex is guided to its mRNA target by the miRNA strand, which typically base pairs imperfectly to its target in the 3′ untranslated region, signaling the target for translational repression or degradation. Global changes of miRNAs are closely involved in neoplastic processes and tumor progression, and differential expression of miRNAs has been described in various tumors [2,3].

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus known to be an etiologic agent of adult T-cell leukemia/lymphoma (ATL) [4]. More than 20 million people are infected with HTLV-1 worldwide, with the highest prevalence in southwest Japan and the Caribbean basin. HTLV-1 infection has a long clinical latency before transforming into ATL. It has a very poor prognosis because tumor cells are usually resistant to conventional chemotherapeutic agents [5,6]. Patients with ATL are typically highly immunocompromised and have frequent severe infections. Tumor cells from most patients with ATL are positive for CD4, CD25, and FOXP3. These immunologic and phenotypic characteristics of ATL are similar to those of regulatory T cells, and it has been suggested that ATL cells may originate from regulatory T cells, which actively suppress activation of the immune system [7].

Recently, some miRNAs have been shown to be involved in ATL [8-13]. However, oncogenetic and clinical significance of miRNAs have not been well clarified. In this study, we examined ATL cell lines and clinical ATL cases for miRNA expression using miRNA arrays, quantitative reverse transcription (RT) polymerase chain reaction (PCR), and the miRNA transfection assay.

2. Materials and methods

2.1. ATL cell lines and normal CD4-positive T cells

The 9 cell lines used in this study were as follows; ATL-102, ATN-1, HUT-102, MJ, MT-1, MT-2, MT-4, TL-Om1, and TL-SU. These cell lines were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen, GIBCO, Carlsbad, CA), at 37°C with 5% CO_2. Mononuclear cells obtained from healthy volunteers (n = 4) were sorted into CD4-positive T cells.

2.2. Patients with ATL

Forty specimens from patients with aggressive ATL of the acute- or lymphoma-type [5] were retrieved from the pathology files of Nagoya City University, Graduate School of Medical Sciences, and Imamura Bun-in Hospital. All specimens were obtained at the initial presentation of the patients and were fixed in formalin and embedded in paraffin. All cases were positive for monoclonal integration of HTLV-1 provirus DNA. The pathological specimens were reviewed according to criteria of the World Health Organization classification of malignant lymphomas [6]. The median age at diagnosis was 60 years (range, 42-88 years), with a male-to-female ratio of approximately 1:1. All cases were within the morphologic boundaries of ATL and exhibited the following immunophenotypes: CD20−, CD3+, CD5+, CD4+, and CD25+. Thirty-three cases were histologically classified as the pleomorphic medium-large cell type, 3 as the pleomorphic small cell type, 3 as the anaplastic type, and 1 as the Hodgkin-like type. All patients were treated with doxorubicin-containing combination chemotherapy regimens. The study was approved by the institutional review board of Nagoya City University.

2.3. miRNA expression array

Using a flash PAGE system (Ambion, Carlsbad, CA), miRNA was extracted from 4 ATL cell lines (ATN-1, HUT-102, MJ, and TL-Om1). For the miRNA microarray, CD4-positive T-cell samples obtained from peripheral blood of 4 healthy volunteers were evenly mixed, and miRNA was extracted and used as a control miRNA. ATL has been suggested to originate from regulatory T cells, which are less than 2% of total CD4-positive cells in peripheral blood [14] and include 2 subsets, naturally occurring and inducible types [15]. Because it was difficult to obtain sufficient numbers of regulatory T cells for the experiment and to induce regulatory T cells specifically, we used CD4-positive T cells as a control in this study. miRNA samples were labeled with Cy-5 using a Label IT miRNA Labeling kit (Mirus Bio, Madison, WI) and hybridized to the array slides (mirVana miRNA Bioarray V2; Ambion). Signals were scanned with an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA), and the miRNA array data thus obtained were analyzed using a Microarray Data Analysis Tool (Filgen, Nagoya, Japan) to select miRNAs significantly up- or down-regulated in all 4 ATL lines compared with control normal CD4-positive T cells.

2.4. miRNA isolation and quantitative RT-PCR in ATL cell lines

Total RNA extracted from fresh ATL cell lines (n = 9) and normal CD4-positive T cells from the 4 healthy volunteers was further miRNA-enriched using a PureLink miRNA

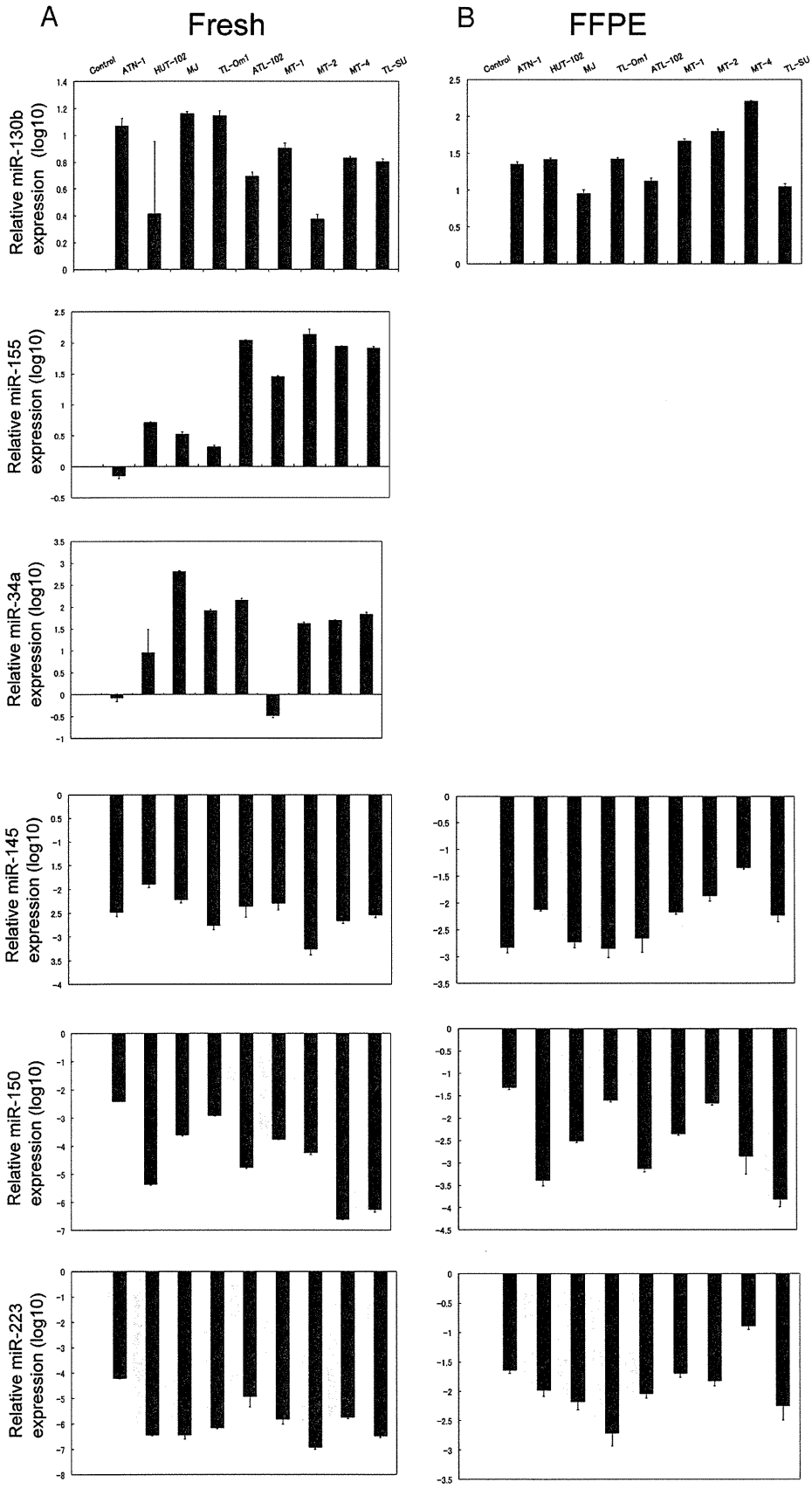

Fig. 1 Relative expression (log 10) of 6 miRNAs (miR-130b, miR-155, miR-34a, miR-145, miR-150, and miR-223) in 9 ATL cell lines (ATN-1, HUT102, MJ, TL-Om1, ATL-102, MT-1, MT-2, MT-4, and TL-SU). The 6 graphs (A) show levels using fresh ATL cell lines, and the 4 graphs (B) show levels using formalin-fixed, paraffin-embedded (FFPE) ATL cell lines.

Isolation kit (Invitrogen, Tokyo, Japan). Quantitative RT-PCR for the targeted mature miRNAs was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a TaqMan MicroRNA Assay kit (Applied Biosystems). All PCR reactions were run in triplicate, and miRNA expression relative to control RNU6B was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of miRNAs in T cells from the 4 healthy individuals was used as a quantitative control. ATL cell lines were fixed in formalin and embedded in paraffin. miRNAs extracted from the paraffin sections were subjected to quantitative RT-PCR to confirm that the target miRNAs were correctly quantified using paraffin sections as well.

2.5. miRNA expression in patients with ATL

Enriched total miRNA was extracted from paraffin-embedded materials of CD4-positive T cells from healthy patients and patients with ATL. Expression of target miRNAs was similarly semiquantified using quantitative RT-PCR. Cutoff values for higher or lower expression of respective miRNAs were set to give superior segregation into prognostic groups in overall survival. For patients with ATL, selected miRNAs were correlated with various clinicopathological factors (age, sex, the presence of B symptoms, extranodal sites, bone marrow involvement, serum lactate dehydrogenase (LDH) values more than twice the normal upper limit [5], histologic lymphoma subtype, and overall survival of the patients).

2.6. miRNA transfection and cell viability and death

MT-4 ATL cells (2×10^6) were resuspended in 100 μL of Nucleofector solution (Cell Line Nucleofector Kit V; Amaxa Biosystems, Cologne, Germany). Cells were electroporated with 100 and 300 nmol/L pre-miR has-miR-145 miRNA precursor, and a pre-miR negative control no. 1 using a Nucleofector (Pre-miR miRNA precursor Starter Kit; Ambion). Transfected cells were plated onto 6-well plates, and 1 mL/well antibiotic-free medium was added for incubation at 37°C with 5% CO_2. After 6 hours, 1.5 mL/well RPMI 1640 media supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin were added. Cells were plated in 96-well plates at 6×10^3 cells/well and cultured for 0, 24, 48, and 72 hours to test their viability using a methyl thiazolyl tetrazorium (MTT). The remaining cells were extracted for miRNA using an miRNA Isolation kit (Invitrogen) to verify electroporation quality.

2.7. Statistical analysis

All statistical analyses were performed using JMP (SAS, Cary, NC). The relationship between miRNA expression and various clinicopathological factors was evaluated using the Mann-Whitney U test or Fisher exact test. Survival curves were plotted using the Kaplan-Meier method, and the Cox proportional hazards model was applied for univariate and multivariate prognostic analysis. The in vitro data were analyzed using a paired t test. A probability value of $P < .05$ was regarded as statistically significant. All tests were 2 tailed.

3. Results

3.1. miRNA array analysis using ATL cell lines

Using miRNA array analysis, we profiled 4 ATL cell lines (ATN-1, HUT-102, MJ, and TL-Om1) using normal CD4-positive T cells as a control. In these 4 ATL cell lines, 3 miRNAs (miR-34a, miR-130b, and miR-155) were consistently highly up-regulated, and 3 miRNAs (miR-145, miR-150, and miR-223) were consistently highly down-regulated (Supplementary Fig. S1). To confirm the results of miRNA array data, we quantified these 6 miRNAs using quantitative real-time RT-PCR with the fresh 9 ATL cell lines (ATN-1, HUT-102, MJ, TL-Om1, ATL-102, MT-1, MT-2, MT-4, and TL-SU), including the 4 ATL cell lines used for miRNA array analysis (Fig. 1A). Of the 6 miRNAs tested, 4 (miR-130b, miR-145, miR-223, and miR-150) were consistently up- or down-regulated in the 9 ATL cell lines (Fig. 1A). Although up-regulated in the miRNA array, miR-34a and miR-155 were down-regulated in 1 and 2 of the 9 ATL cell lines, respectively (Fig. 1A), and we excluded these 2 miRNAs. The remaining 4 miRNAs were precisely quantified by quantitative RT-PCR using formalin-fixed, paraffin-embedded ATL cell lines as well (Fig. 1B).

Table Univariate and multivariate prognostic analyses of miRNAs for overall survival of the patients with ATL

miRNA	Expression	Overall survival					
		Univariate			Multivariate		
		P	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI
miR130b	Higher	.0017	1.83	1.24-2.83	NS		
miR145	Lower	<.0001	3.01	1.79-5.16	.0005	2.59	1.52-4.49
miR150	Lower	NS					
miR223	Lower	.0010	0.96	1.29-3.25	NS		

Abbreviations: CI, confidence interval; NS, not significant.

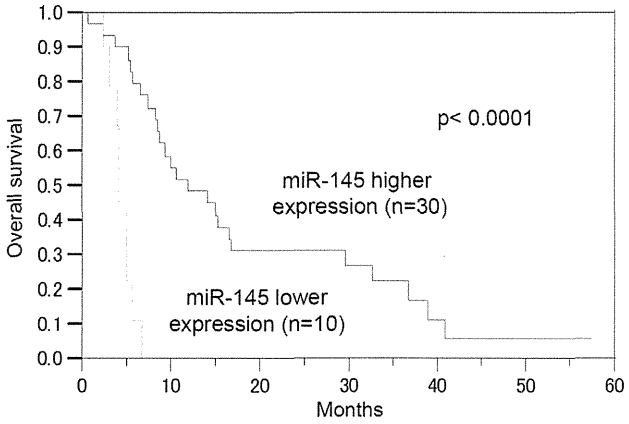

Fig. 2 Overall survival analysis of patients with ATL for miR-145 expression.

3.2. Expression of miRNAs in clinical ATL samples

Using quantitative RT-PCR, 40 clinical ATL cases (paraffin-embedded tumor samples) were examined for expression levels of the selected 4 miRNAs (miR-130b, miR-145, miR-150, and miR-223). To explore miRNAs highly associated with the clinical course, the prognostic impact of the 4 miRNAs on the overall survival of patients with ATL was analyzed. As shown in the Table, using a respective cutoff value that showed superior segregation into prognostic groups, 28 cases were higher expressers for miR-130b ($P = .0017$) and 10 cases each were lower expressers for miR-145 ($P < .0001$) and miR-223 ($P = .0010$). Expression of miR-150 failed to show any prognostic impact. The 3 miRNAs that showed a prognostic impact

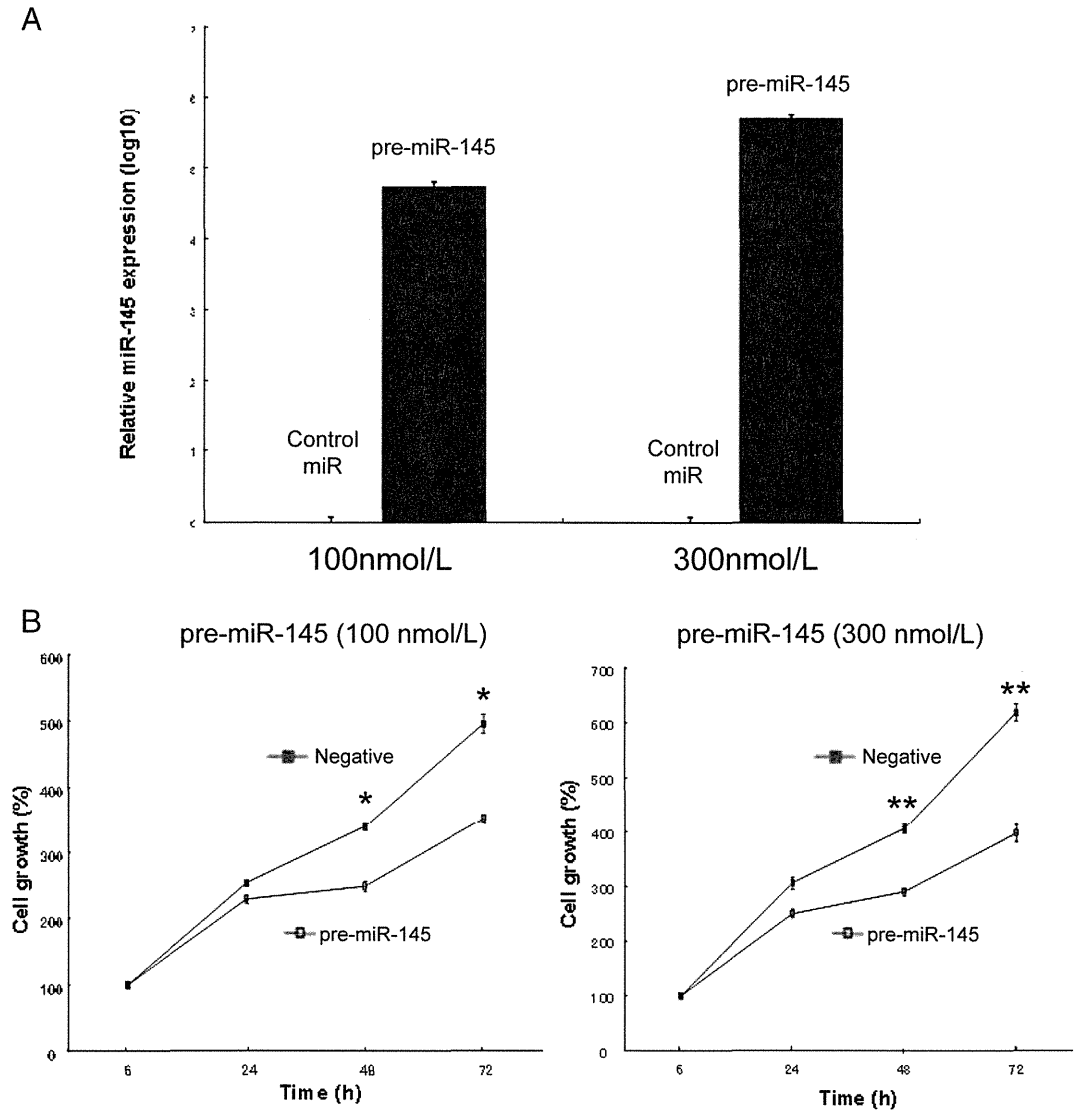

Fig. 3 miR-145 transfection assay. A, Relative expression (log 10) of miR-145 in ATL cells (MT-4) transfected with pre–miR-145 precursor or with negative control pre-miRNA precursor after a 48-hour incubation at a final concentration of 100 (left) or 300 nM (right). B, The effect of pre–miR-145 on growth of ATL cells (MT-4) as determined by an MTT assay. Cell proliferation is significantly inhibited by enforced expression of miR-145. The data represent the means ± SEM of 4 independent experiments. *$P < .05$ and **$P < .01$.