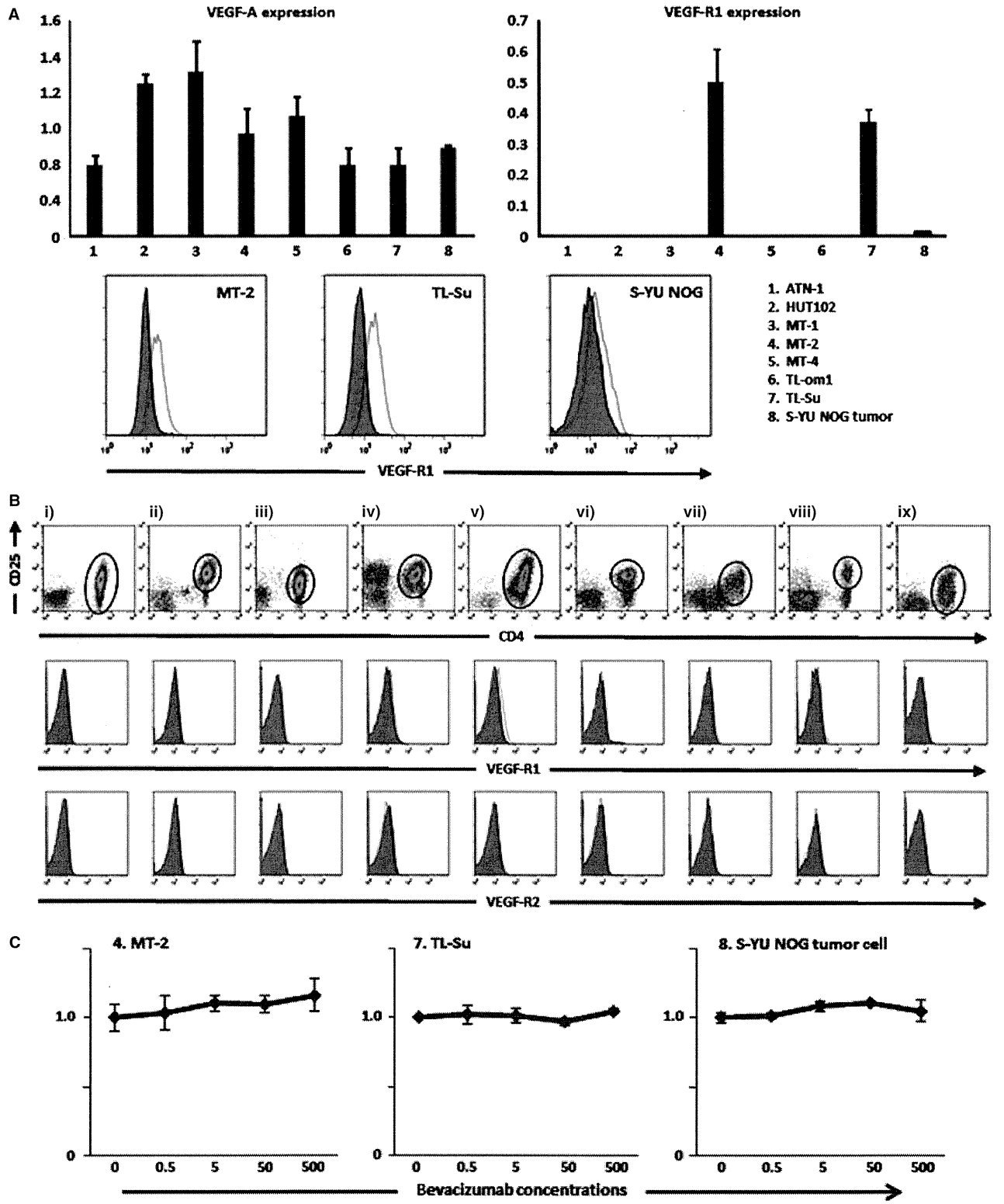

Figure 3 Vascular endothelial growth factor A (VEGF-A), VEGF-R1, and -R2 expression in primary ATL cells, or ATL and HTLV-1-immortalized lines (A) Quantitative RT-PCR analysis for VEGF-A and VEGF-R1 in 7 ATL and HTLV-1-immortalized lines, and NOG ATL cells from the intraperitoneal mass (upper panels). Flow cytometry for VEGF-R1 in HTLV-1-immortalized lines MT-2 and TL-Su, and NOG ATL cells, from the intraperitoneal mass (lower panels). (B) Flow cytometry for VEGF-R1, and -R2 in 9 primary ATL cells. (C) Bevacizumab has no direct anti-proliferative activity against HTLV-1-immortalized lines (MT-2 and TL-Su) expressing both VEGF-A and VEGF-R1, or NOG ATL cells, *in vitro*. Each result represents three independent experiments.

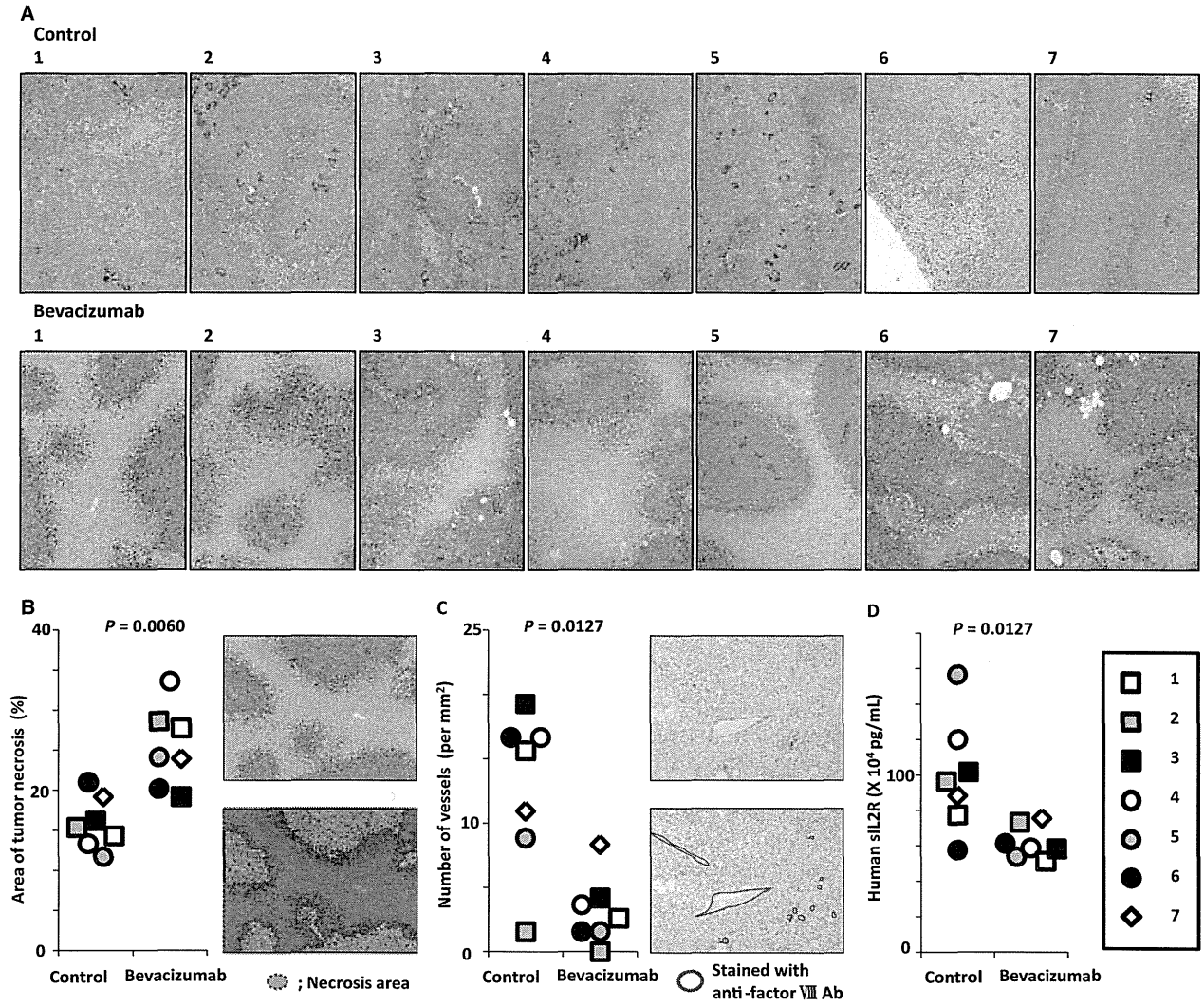

Figure 4 Bevacizumab therapy has significant therapeutic efficacy in the ATL cell-bearing NOG mouse model. (A) Macroscopic photomicrographs with hematoxylin and eosin staining of mice given saline (control) (upper panels) or bevacizumab (lower panels). (B) Area of tumor necrosis (%) of each ATL cell-bearing NOG mouse. The bevacizumab-treated mice had significantly greater tumor necrosis than control mice (left panel). An example of a calculation for tumor necrosis area (%) by means of Image J software is shown (right panels). (C) Numbers of vessels (/mm^2) of each ATL cell-bearing NOG mouse. The bevacizumab recipients had significantly fewer vessels than controls (left panel). An example of such a calculation by means of Image J software is shown (right panels). (D) Serum sIL2R concentrations of each ATL cell-bearing NOG mouse. The bevacizumab recipients had significantly lower levels of sIL2R than controls.

the tumor burden of the human CD25-expressing ATL (26). Treatment with bevacizumab showed significantly greater therapeutic efficacy as demonstrated by sIL2R concentrations in S-YU cell-bearing NOG mice (617.9, 588.5, 513.2–755.7 $\times$ 10^3 pg/mL), compared to controls (996.6, 963.4, 575.7–1565.0 $\times$ 10^3 pg/mL, P = 0.0127) (Fig. 4D). Although bevacizumab monotherapy showed significant therapeutic efficacy as demonstrated by the percentage of tumor necrosis, vascular number in the tumor tissues, and sIL2R concentrations in sera (Fig. 4), it did not confer any survival advantage to the NOG/S-YU mice (Fig. 5A). No toxicity attributable to bevacizumab injections was observed in any of the mice in this setting.

Therapeutic efficacy of bevacizumab plus CHOP compared to CHOP alone in S-YU cell-bearing NOG mice

The bevacizumab plus CHOP group did have a significant prolongation of survival compared with CHOP alone (P = 0.046). The median survival time of bevacizumab plus CHOP and CHOP alone was 38 and 34 d, respectively.

Discussion

In this study, we have demonstrated that bevacizumab possesses significant therapeutic efficacy in an ATL mouse model in which the tumor cells from a patient survive and

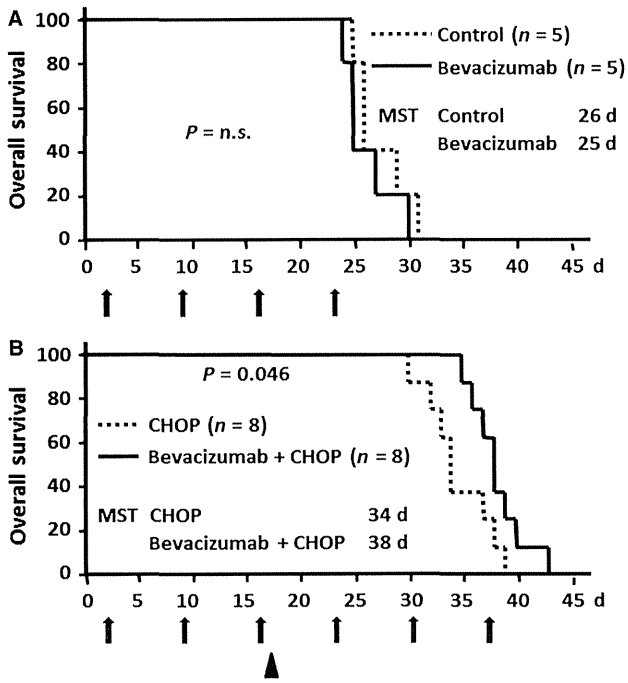

Figure 5 Survival analysis of ATL cell-bearing NOG mice treated with bevacizumab (A) Kaplan–Meier survival curves of ATL cell-bearing NOG mice treated with bevacizumab or saline. Arrows, bevacizumab or control (saline) injections. Each group consists of five mice. The difference between the bevacizumab and control groups is not significant. (B) Kaplan–Meier survival curves of ATL cell-bearing NOG mice treated with bevacizumab + CHOP, or CHOP alone. Arrows, bevacizumab or control (saline) injections. Arrow head, CHOP injection. Each group consists of eight mice. The difference between the bevacizumab + CHOP and CHOP alone is statistically significant.

proliferate in a murine microenvironment-dependent manner. The present finding revealed the importance of angiogenesis for the pathogenesis of VEGF-expressing ATL.

NOG mice have severe, multiple immune defects, such that human immune cells engrafted into them retain essentially the same functions as in humans (27, 28). While it has been reported that S-YU cells can be serially transplanted into SCID mice as recipients, the present study demonstrated that S-YU cells could also be serially transplanted into NOG mice. This was not unexpected given the even more severe immune dysfunction of NOG mice compared to SCID mice. This may also explain why the ATL tumor masses were much larger in NOG than in SCID mice.

In this study, most primary ATL cases (22/23), and all of the established cell lines tested (7/7), were positive for VEGF-A. These results are consistent with data from other investigators (16, 18, 19). Thus, the VEGF-A produced by ATL cells is likely to play an important role in the pathogenesis of ATL. On the other hand, *VEGF-R1 mRNA* expression was only seen in two of the seven ATL and HTLV-1-immortalized lines, and *VEGF–R2* in none of them. VEGF-R1 protein expression by primary ATL tumor cells was only seen in one of nine patients, and VEGF-R2 in none. In B-cell lymphomas, an earlier study reported that tumor cell growth was promoted in an autocrine fashion via VEGF-A/VEGF-R1 or VEGF-A/VEGF-R2 interactions (29). However, the present analysis of VEGF-R1/R2 expression in ATL, and the results of *in vitro* proliferation assays, did not support the existence of such an autocrine loop in ATL.

Because S-YU cells can only be maintained by serial transplantation in immunodeficient mice, but not by *in vitro* culture (30), the microenvironment is likely to be indispensable for their survival. S-YU are positive for VEGF-A, and therefore it would be expected that the interaction of VEGF-A produced by ATL cells with receptors on host (murine) endothelial cells should play an important role in tumor angiogenesis. This would lead to tumor cell survival and proliferation supported by transport of sufficient nutrients and oxygen in the mouse. Therefore, the present ATL model using S-YU should better reflect the human ATL *in vivo* environment, compared to other mouse models using established ATL cell lines, or HTLV-1-immortalized lines. Thus, this model should provide a powerful tool for understanding the pathogenesis of ATL. Furthermore, it should be useful not only for evaluating novel cytotoxic anti-ATL agents, but also provide a more appropriate *in vivo* model to test antitumor agents targeting the microenvironment, including bevacizumab.

The effect observed in mice receiving bevacizumab monotherapy, as demonstrated by the increased tumor necrosis area and reduced vasculature in the tumor tissue, was expected, given the conventional antitumor mechanism of bevacizumab, which neutralizes the human VEGF-A produced by the tumor cells, but not murine VEGF-A (31). It then inhibits the growth of new blood vessels and thus starves tumor cells of necessary nutrients and oxygen (32). This should lead to a reduced tumor burden, as indicated by the sIL2R concentrations measured. Although bevacizumab monotherapy did show this anti-angiogenesis effect, it did not lead to survival prolongation in this study. This finding is consistent with the clinical observations in many types of cancer such as colorectal cancer, non-small-cell lung cancer, renal cell carcinoma, and ovarian cancer. On the other hand, combination treatment with bevacizumab and CHOP did prolong survival compared to CHOP alone. Nonetheless, the extent of this prolongation was not marked, which is also consistent with clinical observations in many types of cancer where bevacizumab is of limited benefit and then only when combined with chemotherapy. This study suggested that the tumor cell 'starvation effect' alone mediated by bevacizumab does not result in prolonged survival. It has been reported that VEGF-targeted therapy can 'normalize' the tumor vascular network and that this can lead to a more uniform blood flow, with subsequent increased delivery of chemotherapeutic agents (33, 34). This normalization by bevacizumab is a possible explanation for the prolonged survival in the present combination setting.

The present study demonstrated the importance of angiogenesis for the pathogenesis of ATL and the potential efficacy of blocking this in at least a subgroup of patients with ATL. In recent clinical cancer therapy experience, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, gefitinib, failed to yield significantly improved overall survival in patients with refractory NSCLC, but did show therapeutic benefit in a subgroup of patients with mutated EGFR (35). In the case of mAb targeting the EGFR, both panitumumab and cetuximab also yield clinical benefits only in a subgroup of colorectal cancer patients with wild-type *KRAS* and BRAF (36). These findings indicate that we should develop novel treatment strategies based on tumor biology, and not on tumor category. Therefore, as a next step, further investigations are warranted to determine which subgroups of patients with ATL will benefit from bevacizumab therapy (37). In other words, we should face the challenge of developing robust biomarkers that can guide selection of those patients with ATL for whom bevacizumab therapy will be most beneficial. In addition, several promising new agents for treating ATL are currently being developed (1, 38–40). Investigations of combinations of bevacizumab with these novel agents are also warranted.

In conclusion, to the best of our knowledge this is the first report to evaluate the efficacy of bevacizumab for ATL in a tumor microenvironment-dependent animal model. Bevacizumab therapy combined with chemotherapy could be a potential treatment strategy for that subgroup of patients with ATL probably depending to a large extent on angiogenesis via VEGF for tumor survival and proliferation.

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Authorship contributions

Mori F, Ishida T, Asahi I, and Ueda R designed the research. Mori F, Ishida T, Asahi I, Sato F, Masaki A, Narita T, Suzuki S, Yamada T, and Takino H performed the research. Hishizawa M, Imada K, Takaori-Kondo A contributed to establishing the ATL mouse model. All the authors analyzed the data and wrote the article.

Conflict of interest

Nagoya City University Graduate School of Medical Sciences has received research funding for Takashi Ishida, Shigeru Kusumoto, and Shinsuke Iida from Chugai Pharmaceutical Co., Ltd. The other authors have no financial conflicts of interest related with this study.

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Over one-third of African-American MGUS and multiple myeloma patients are carriers of hyperphosphorylated paratarg-7, an autosomal dominantly inherited risk factor for MGUS/MM

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As hyperphosphorylated paratarg-7 (pP-7) carrier state was shown to be the first molecularly defined autosomal dominantly inherited risk factor for monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM) in a European population, the prevalence of pP-7 carrier state among African-Americans who have a significantly higher incidence of MGUS/MM is of interest. We therefore determined pP-7 carrier state and paraproteins with specificity for P-7 in African-American, European and Japanese patients with MGUS/MM and healthy controls. By isoelectric focusing and ELISA, a paratarg-7-specific paraprotein and the associated pP-7 carrier state was observed in 30/81 (37.0%) African-American, 42/252 (16.7%) European and 7/176 (4.0%) Japanese MGUS/MM patients ($p < 0.001$). A pP-7 carrier state was found in 11/100 (11.0%) African-American, 8/550 (1.5%) European and 1/278 (0.4%) Japanese healthy controls ($p < 0.001$), resulting in an odds ratio for MGUS/MM of 4.8 ($p < 0.001$) among African-American, 13.6 among European ($p < 0.001$) and 11.5 ($p = 0.023$) among Japanese carriers of pP-7. We conclude that pP-7 carriers are most prevalent among African-Americans, but a pP-7 carrier state is the strongest molecularly defined single risk factor for MGUS/MM known to date in all three ethnic groups. The high prevalence of pP-7 carriers among African-American patients emphasizes a predominant role of this genetic factor in the pathogenesis of these diseases. The large number of pP7 African-American patients and controls should facilitate the identification of the SNP or mutation underlying the pP-7 carrier state.

Epidemiology

A causal relationship between monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM) and chronic antigenic stimulation has been suggested by the results of several studies[1]; hence, the identification of the antigenic stimuli of B-cell neoplasms is of interest. In a modification of SEREX[2] using a commercially available human fetal brain-derived protein macroarray and IgA or IgG paraprotein-containing sera, paratarg-7 was shown to be a frequent antigenic target of paraproteins from European patients with MGUS, MM and Waldenstrom's macroglobulinemia.[3–5] All patients with paratarg-7-specific paraproteins were carriers of a hyperphosphorylated version of the protein (pP-7) and this hyperphosphorylation is inherited in a dominant fashion.[3–5] The hyperphosphorylation of paratarg-7 in these patients is due to an inactivation of protein phosphatase 2A resulting in the failure to dephosphorylate the physiologically occurring phosphorylation of p-7 at serine 17.[6] Paratarg-7 is identical to STOML2 (stomatin [EPB72]-like), also known as HSPC108 or stomatin-like-protein and SLP-2,[7] which is expressed in all human tissues.[8] The physiological function of SLP-2 is unknown. As few healthy Europeans are carriers of pP-7, pP-7 carrier state is associated with an

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What's new?

African-Americans are more than twice as likely as the general population to develop monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM). A hyperphosphorylated form of the protein paratarg-7, called pP-7, is a dominantly inherited risk factor for MGUS/MM. In this study, the authors found that more than one third of African-American MGUS/MM patients expresspP-7. This suggests that pP-7 might play a role in the pathogenesis of MGUS/MM, and should facilitate the identification of the SNP or mutation responsible for the pP-7 carrier state.

increased risk for MGUS/MM. Thus, pP-7 is the first molecularly defined inherited risk factor for any hematological neoplasm known to date. The incidence of MGUS and MM is lower in Asians and higher in African-Americans than in Europeans[9,10]; therefore, we compared these three ethnic groups with respect to the prevalence of a pP-7 carrier state and the incidence of P-7-specific paraproteins in MGUS/MM and healthy controls.

Material and Methods

Patients and controls

This study was approved by the local ethical review boards of the participating institutions. Consecutive patients with MGUS/MM with an IgA, IgD or IgG paraprotein were included. Healthy European, Japanese and African-American blood donors served as controls. Healthy was defined as having no monoclonal immunoglobulin by serum electrophoresis and immunofixation, and being healthy as diagnosed by the Medical Officer in the pre-donation check-up. Samples of African-American MGUS/MM patients and patients with other malignant diseases were collected at Emory University Hospital in Atlanta, GA, as were healthy African-American controls. Autoimmune-disease blood samples were obtained from patients diagnosed and treated at the Autoimmune Disease Clinics of the Department of Internal Medicine I, Saarland University Medical School. Written informed consent was obtained from all patients and controls. Peripheral blood was centrifuged, and plasma and cells were stored at −20°C.

Isoelectric focusing for the determination of the pP-7 carrier state

The isoelectric focusing to determine the pP-7 carrier state was performed as described before.[4,6,11]

Paratarg-7 ELISA for the detection of paraproteins with specificity to paratarg-7

The paratarg-7 ELISA using full-length recombinant paratarg-7 was performed as described previously.[3]

Statistical methods

Odds ratios with 95% confidence intervals (CIs) and p-values are presented to describe the risk for MGUS/MM (in relation to healthy controls) in pP-7 carriers separately for each of the three ethnic groups. To compare the odds ratios between ethnic groups Breslow–Day tests were performed. Chi-square and if necessary Fisher's exact tests were used to test differences between ethnic groups regarding the prevalence of pP-7, separately for MM/MGUS patients and healthy controls. In case of significant global test over all three ethnic groups pairwise tests were performed. For comparison of prevalence of pP-7 carriers among healthy controls and MGUS/MM patients within each of the ethnic groups we used chi-square and if necessary Fisher's exact tests. For differences regarding patient characteristics, we calculated chi-square and if necessary Fisher's exact tests for qualitative data and Wilcoxon rank sum tests for quantitative data (Table 2). The significance level was $p = 0.05$. Statistical analyses were done with IBM SPSS Statistics 20.

Results

A total of 252 European, 176 Japanese and 81 Afro-American MGUS and MM patients were included in this study. About 30/81 (37.0%) African-American, 42/252 (16.7%) European and 7/176 (4.0%) Japanese MGUS/MM

Table 1. Detection of paratarg-7 specific paraproteins in consecutive African-American, European and Japanese patients with MGUS/MM

	MGUS (%)	MM (%)	Total (%)
African-Americans			
IgA	2/6 (33.3)	2/4 (50)	4/10 (40.0)
IgD	0/0	0/1	0/1
IgG[1]	8/22 (36.4)	18/42 (42.9)	26/64 (40.6)
Light chain	0/0	0/6	0/6
Total	10/28 (35.7)	20/53 (37.7)	30/81 (37.0)
Europeans			
IgA	1/7 (14.3)	4/24 (16.7)	5/31 (16.1)
IgD	0/0	0/0	0/0
IgG[2]	5/45 (11.1)	32/176 (18.2)	37/221 (16.7)
Total	6/52 (11.5)	36/200 (18.0)	42/252 (16.7)
Japanese			
IgA	0/4	1/32 (3.1)	1/36 (2.8)
IgD	0/0	0/11	0/11
IgG[2]	0/13	6/116 (5.2)	6/129 (4.7)
Total	0/17	7/159 (4.4)	7/176 (4.0)

[1]All pP-7 reactive IgG paraproteins were of the IgG_3 subclass except 1 IgG_1.
[2]All pP-7 reactive IgG paraproteins were of the IgG_3 subclass.

Epidemiology

Table 2. Characteristics of African-American MGUS and MM patients who are carriers of hyperphosphorylated paratarg-7 and those who are not

	pP7$^+$ n = 30 (%)	pP7 n = 51 (%)	p-value[1]
Age (years)			
Median	64	64	0.325
Range	43–89	32–84	
Diagnosis			
MGUS	10 (33.3)	17 (33.3)	1.000
Multiple Myeloma	19 (63.3)	32 (62.7)	0.958
Stage I	3 (15.8)	3 (9.7)	0.778[2]
Stage II	7 (36.8)	11 (35.5)	
Stage III	9 (47.4)	17 (54.8)	
Unknown		1 (3.1)	
Plasmacytoma	1 (3.3)	2 (3.9)	1.000
Paraprotein			
IgG	26 (86.7)	38 (74.5)	0.194
IgG_1	1 (3.3)	23 (45.1)	<0.001
IgG_2	0 (0.0)	0 (0.0)	–
IgG_3	25 (83.3)	15 (29.4)	<0.001
IgG_4	0 (0.0)	0 (0.0)	–
IgA	4 (13.3)	6 (11.8)	1.000
IgD	0 (0.0)	1 (2.0)	1.000
Light chain	0 (0.0)	6 (11.8)	0.080
Lytic lesions			
Yes	14 (48.3)	27 (52.9)	0.688[3]
No	15 (51.7)	24 (47.1)	
Unknown		1 (3.3)	
Hemoglobin (g/dl)			
Median	11.1	11.3	0.491
Range	7.2–14.9	6.7–15.8	
Creatinine (mg/dl)			
Median	1.1	1.4	0.082
Range	0.7–5.0	0.6–20.0	
Calcium (mg/dl)			
Median	10.0	9.4	0.221
Range	2.9–13.0	7.0–14.0	
ß2 microglobulin (mg/l)			
Median	3.0	3.6	0.637
Range	2.0–31.6	1.5–19.9	
Cytogenetics			
Normal	10 (33.3)	25 (49.0)	0.169
Trisomy 3	1 (3.3)	0 (0.0)	0.370
Trisomy 8	0 (0.0)	1 (2.0)	1.000
Complex	12 (40.0)	12 (23.5)	0.117
Unknown	7 (23.3)	13 (25.5)	

[1]For qualitative data chi-square test and if necessary Fisher's exact test and for quantitative data Wilcoxon rank sum test were used.
[2]p-value for 'Stages I–III'.
[3]p-value without 'unknown'.

Table 3. Prevalence of pP-7 carrier state in African-American patients with hematological neoplasms other than MGUS/MM

Acute lymphocytic leukemia	**0/2**
Acute myeloid leukemia	0/4
Chronic lymphocytic leukemia	2/13
Chronic myeloid leukemia	2/6
Diffuse large B-cell lymphoma	2/24
Follicular lymphoma	0/7
Hodgkin lymphoma	0/8
Lymphomas, others	2/8
Total	8/72

Table 4. Prevalence of pP-7 carrier state in European patients with autoimmune diseases

Autoimmune disorder	
- Rheumatoid arthritis	1/100
- Systemic lupus erythematosus	0/30
- Polymyalgia rheumatic	0/15
- Granulomatosis with polyangiitis	0/10
- Multiple sclerosis	0/10
- Crohn's disease	0/10
- Churg–Strauss syndrome	0/5
- SAPHO syndrome	0/5
- Sjögren's syndrome	0/5
Total autoimmune disorders	1/190 (0.5%)
European healthy controls	8/550 (1.5%)

patients had a paraprotein reacting with paratarg-7. All these patients were pP-7 carriers (Table 1). Notably, the 30 African-American patients with a pP-7 specific paraprotein was the first among the total of 69 patients with an IgG paraprotein who did not belong to the IgG_3, but rather to the IgG_1 subtype. The characteristics of African-American MGUS/MM patients with p-7 specific paraproteins and with paraproteins that did not bind to paratarg-7 showed no significant differences (Table 2). The differences between ethnic groups regarding the prevalence of pP-7 among MM/MGUS patients were significant ($p < 0.001$) for the global test, as were the differences between African-American (37.0%) and European (16.7%; $p < 0.001$), African-American and Japanese (4.0%; $p < 0.001$) and European and Japanese patients ($p < 0.001$).

The prevalence of healthy pP-7 carriers was 11/100 (11.0%) among healthy African-Americans, 8/550 (1.5%) in Europeans and 1/278 (0.4%) in Japanese ($p < 0.001$ in the global test). The prevalence of pP-7 carriers in the European and Japanese controls was not different ($p = 0.286$), but the prevalence of healthy pP-7 carriers in the African-Americans were significantly higher than in the European and Japanese population ($p < 0.001$ and $p < 0.001$), respectively.

The prevalence of pP-7 carriers was lower in healthy controls than in MGUS/MM patients in all three ethnic groups (African-Americans: 11.0% vs. 37.0%, $p < 0.001$; Europeans: 1.5% vs. 16.7%, $p < 0.001$; Japanese 0.4% vs. 4.0%, $p = 0.007$) resulting in an elevated risk for MGUS/MM among healthy pP-7 carriers (odds ratio: African-Americans: 4.8 [95% CI: 2.2–10.3], $p < 0.001$; Europeans: 13.6 [95% CI: 6.3–29.3], $p < 0.001$; Japanese 11.5 [95% CI: 1.4–94.1], $p = 0.023$). Thus, pP-7 carrier state is the strongest molecularly defined single risk factor for MGUS/MM known to date in all three ethnic groups. The p-value for differences in the odds ratios for MGUS/MM among healthy pP-7 carriers was 0.058 between African-Americans and Europeans, 0.430 between African-Americans and Japanese and 0.884 between Europeans and Japanese.

Discussion

This study is the first that investigated pP-7 carrier state in African-American patients. The high prevalence of pP-7 carriers among African-American MGUS/MM patients is astonishing and intriguing. The frequency of paratarg-7 as a paraprotein target in more than one-third of all African-American MGUS/MM patients suggests a role of pP-7 in the pathogenesis of these diseases in all three ethnic groups. The fact that carriers of pP-7 are only at a higher risk for MGUS/MM and Waldenstrom's macroglobulinemia (which was not included in this study due to the very low incidence of Waldenstrom's macroglobulinemia in African-Americans), but not for other malignancies in neither the European[5] nor the African-American population (Table 3) suggests that the contribution of pP-7 to the pathogenesis is more likely to be mediated by chronic antigenic stimulation and not due to a higher intrinsic potential of cells carrying the hyperphosphorylated paratarg-7 for malignant transformation. This is also supported by the fact that while all 79 patients included in this study with a paraprotein reacting with paratarg-7 were carriers of the hyperphosphorylated version of paratarg-7, none of the 430 MGUS/MM patients with paraproteins not reacting with paratarg-7 were carriers of pP-7.

Notably, the autoimmunity in pP-7 carries appears to be specific for P-7, because we found no increased frequency of pP-7 carriers among 190 patients with autoimmune disease (Table 4).

We do not know the age of the healthy blood donors, but presumably they were considerably younger than the patients. The younger age of the donors does not affect the prevalence data, because pP-7 carriership is inherited and present from birth to death. However, some of the healthy donors might develop MGUS/MM later in their life, but this would only increase the odds ratios for MGUS/MM among healthy pP-7 carriers. We also checked the healthy carriers for anti-P-7 antibodies, because we would expect a polyclonal anti-P-7 reactivity followed by the appearance of an immortalized clone. However, so far we have not (yet) detected such a polyclonal anti-paratarg-7 reactivity in the sera of healthy pP-7 carriers.

Although the differences in the risk for MGUS/MM failed to become significant between the three ethnic groups due to the limited number of patients, there was a strong trend ($p = 0.058$) for a difference between African-Americans and Europeans, which was weaker for the smaller number of African-American and Japanese probands. The reasons for the different risk ratios to develop MGUS/MM in patients of different ethnic background remain to be determined, and might be due to either environmental or additional genetic factors. In this respect, studies of African populations from Africa or Japanese from Hawaii would be very interesting, but even though we tried hard, we got no access to such populations.

We expect the risk of a pP-7 carrier in the family of a pP-7 patient to be even considerably higher, but the number of families with multiple cases of pP-7 MGUS/MM in our database is (still) too small to prove this with a solid statistics. Nevertheless, because two robust tests are available (IEF for the identification of a pP-7 carrier state and ELISA for the detection of paraproteins with specificity for paratarg-7), the identification of family members from a pP-7 MGUS/MM patient at risk, i.e., carriers of pP-7 is an easy task.

With the frequency of pP-7 carriers among healthy African-Americans and patients with MM/MGUS, sufficient numbers of pP-7 MGUS/MM patients and healthy wtP-7 and pP-7 family members should now be available to scrutinize tumour–host interactions in the presence and absence of the antigenic pP-7 in these individuals. Most importantly, the large number of African-American pP-7 carriers both among MGUS/MM patients and healthy controls should now facilitate the dissection of the break-down of tolerance against the autoantigenic pP-7 and the identification of the SNP or mutation responsible fore the inactivation of PP2A, which causes and maintains the hyperphosphorylation of paratarg-7 in individuals with a pP-7 carrier state.[6]

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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

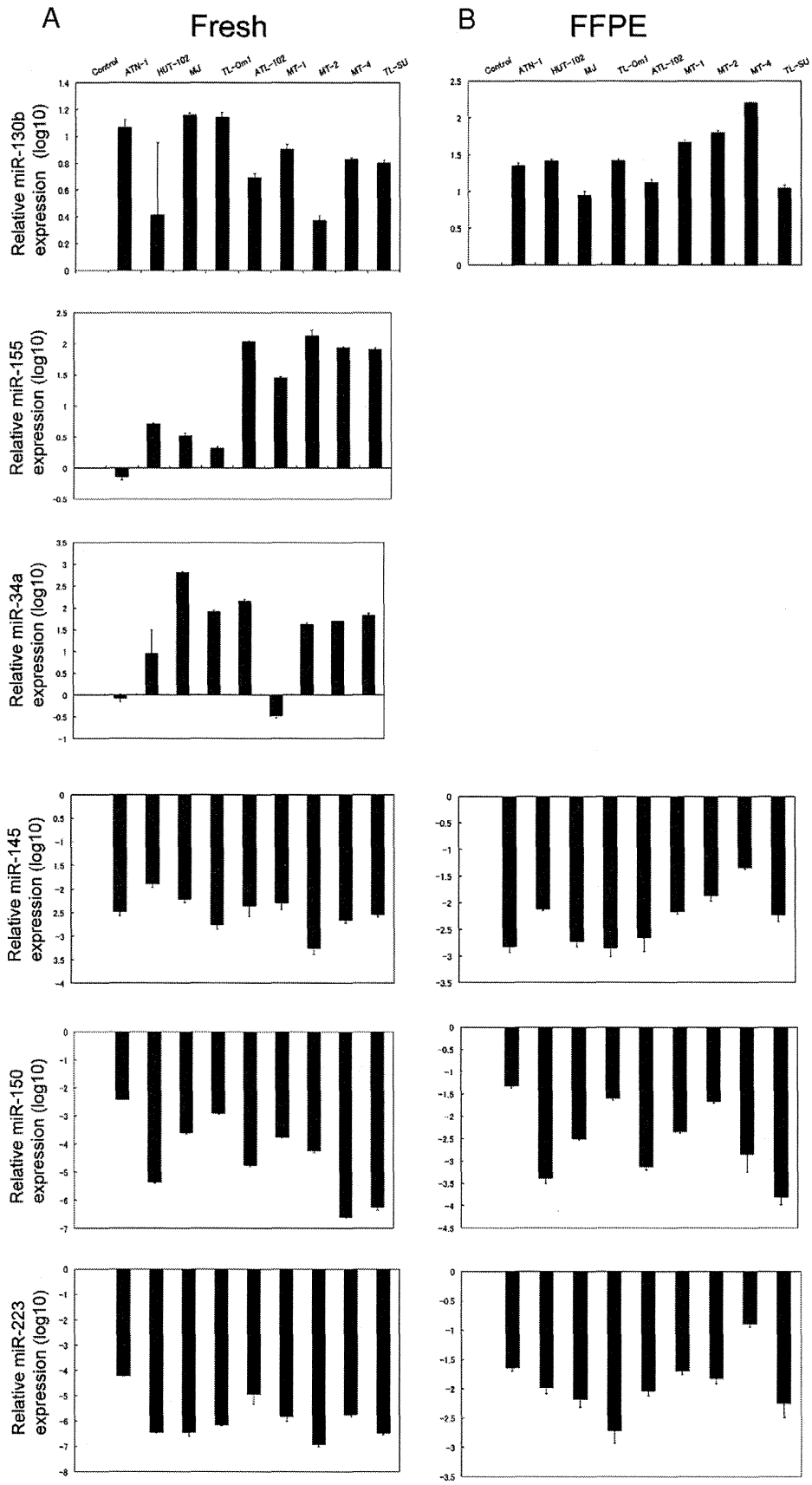

Fig. 1 Relative expression (log 10) of 6 miRNAs (miR-130b, miR-155, miR-34a, miR-145, miR-150, and miR-223) in 9 ATL cell lines (ATN-1, HUT102, MJ, TL-Om1, ATL-102, MT-1, MT-2, MT-4, and TL-SU). The 6 graphs (A) show levels using fresh ATL cell lines, and the 4 graphs (B) show levels using formalin-fixed,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

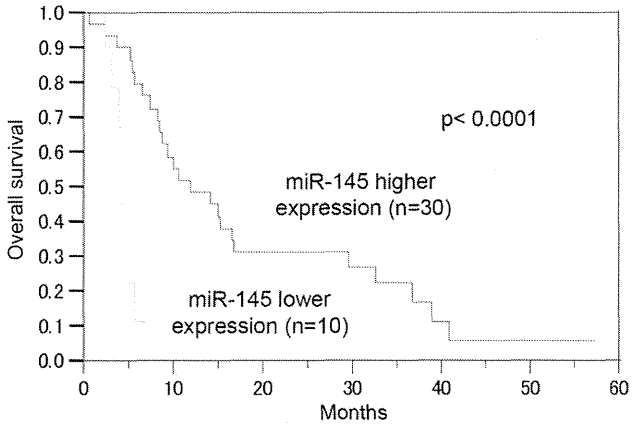

Fig. 2 Overall survival analysis of patients with ATL for miR-145 expression.

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

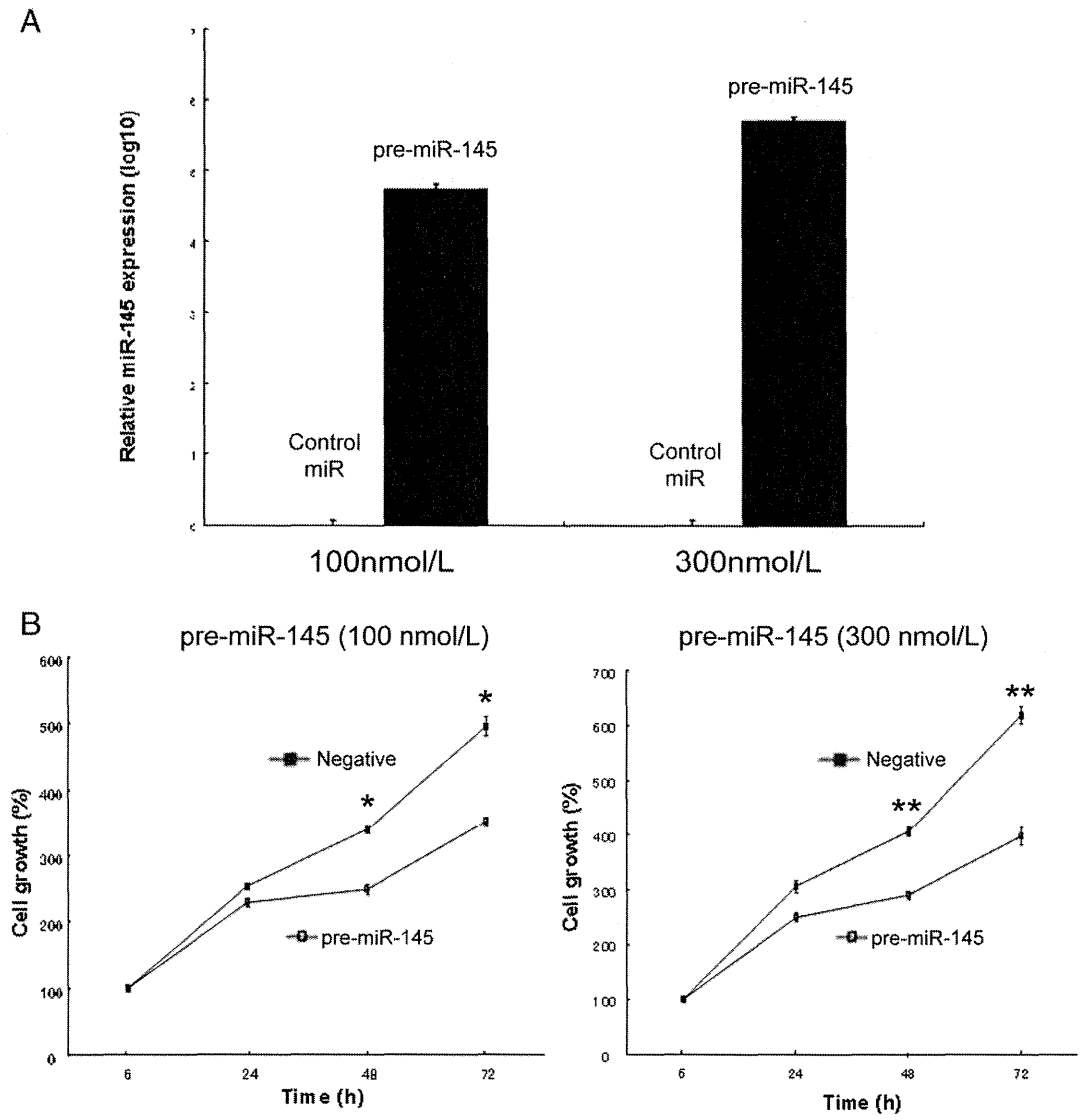

Fig. 3 miR-145 transfection assay. A, Relative expression (log 10) of miR-145 in ATL cells (MT-4) transfected with pre–miR-145 precursor or with negative control pre-miRNA precursor after a 48-hour incubation at a final concentration of 100 (left) or 300 nM (right). B, The effect of pre–miR-145 on growth 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 **$P < .01$.

were found to be associated with each other (miR-130b versus miR-145, $P = .019$; miR-130b versus miR-223, $P < .0001$; and miR-145 versus miR-223, $P = .043$). To explore which miRNA was the most associated with the patients' prognosis, we performed a multivariate analysis. Only miR-145 lower expression was selected as a prognostic factor ($P = .0005$; hazard ratio, 2.59 [1.52-4.49]), whereas the other 2 miRNAs failed to achieve statistical significance (Table). Fig. 2 shows the overall survival curve of patients with ATL for miR-145 expression. miR-145 expression of patients with ATL relative to that of normal CD4-positive T cells was 0.0495 ± 0.0276 and 3.02 ± 1.03 (mean ± SEM) for lower and higher expressers, respectively. When correlated with clinicopathological factors including age, sex, the presence of B symptoms, performance status, extranodal sites, bone marrow involvement, elevated LDH value, and histologic lymphoma subtype, miR-145 lower expression was not associated with any of these factors.

3.3. Overexpression of miR-145 and cell growth inhibition in ATL cells

Because miR-145 was consistently down-regulated in ATL cell lines and showed the highest prognostic impact on patients' survival, we focused on this miRNA in further analysis. To examine the association between miR-145 expression and the growth of ATL cells, MT-4 cells were transfected with a pre–miR-145 precursor and a pre-miR negative control, and an MTT assay was conducted. Overexpression of mature miR-145 in the transfected cells was confirmed by quantitative RT-PCR (Fig. 3A). Cell growth was significantly inhibited in cells transfected with pre–miR-145 48 hours after the transfection by approximately 40% as compared with those transfected with the pre-miR negative control (Fig. 3B). These results indicated that miR-145 specifically inhibited the cell growth of the MT-4 cell line.

4. Discussion

In this study, we found that 1 miRNA (miR-130b) was consistently up-regulated, and 3 (miR-145, miR-223, and miR-150) were consistently down-regulated in ATL cell lines using an miRNA array and subsequent quantitative RT-PCR. It has been shown that expression of some of these miRNAs is dysregulated in ATL cell lines: miR-130b and miR223 have been reported to be up-regulated [8] and down-regulated [10], respectively. miR155, which was selected in the present miRNA array but was subsequently excluded because of its inconsistent expression in the quantitative RT-PCR, has been frequently up-regulated in ATL [9,10,13]. It is difficult to explain this discrepancy. A similar observation was reported in cutaneous T-cell lymphoma, and it was speculated that cross-hybridization of pre–miR-155 to the hybridization probe might have masked the signal of the mature miR-155 [16]. Recently, Yamagishi et al [11] and Tomita et al [12] determined the miRNA signatures and revealed miR-31 down-regulation and miR-146a up-regulation in primary ATL cells, respectively. However, these 2 miRNAs were not highly dysregulated in our miRNA array. This is partly explained by our use of the ATL tumor samples and control samples. For the ATL tumor samples and controls, we used ATL cell lines and normal CD4-positive lymphocytes while Yamagishi et al used clinical ATL cells as tumor samples [11] and Tomita et al used HTLV-1–uninfected T-cell lines as a control [12].

Of the 4 dysregulated miRNAs we determined (miR-130b, miR-145, miR-150, and miR-223), we searched for miRNAs that had a prognostic impact on clinical ATL cases. For each of the 4 miRNAs, we divided our ATL cases into 2 groups (higher and lower expression) using a respective cutoff value that showed superior segregation into prognostic groups. Univariate prognostic analysis showed that miR-130b, miR-145, and miR-223, but not miR-150, had a significant association with the overall survival of patients with ATL. The reason why miR-150 did not achieve statistical significance is difficult to discern, but may be partly explained as follows. First, some miRNAs are expressed differently in ATL cell lines compared with clinical samples, and miR-150 was reported to be differentially expressed in ATL cell lines and uncultured ATL cells [9]. Second, miR-150 may be more associated with tumor development than tumor progression.

We found that the expressions of 3 selected miRNAs (miR-130b, miR-145, and miR-223) were significantly associated with each other. Multivariate prognostic analysis including these 3 miRNAs revealed that only miR-145 lower expression achieved statistical significance. Interestingly, this miRNA was not correlated with any of the clinicopathological or risk factors examined, suggesting that miR-145 lower expression might be a useful independent prognostic factor. Although down-regulated in all ATL cell lines examined, miR-145 was not always down-regulated in patients with clinical ATL. One possible explanation may be that ATL cell line tumor cells are highly activated and more aggressive than clinical ATL cases.

We then focused on miR-145, and to confirm whether miR-145 expression was inversely associated with ATL cell proliferation, we performed an MTT assay to measure cell proliferation rates before and after enforced miR-145 expression in an ATL cell line, MT-4, and showed that overexpression of miR-145 significantly inhibited tumor cell growth. This inhibition was not complete, suggesting that some other factors or pathways may be involved in tumor cell growth. Down-regulation of miR-145 has been reported in various types of human carcinoma [17-20] and B-cell malignancy [21-23]. These studies suggest that miR-145 plays a role in controlling cell proliferation, thereby serving as a tumor suppressor. The precise upstream mechanism of miR-145 down-regulation in ATL has not been clarified.

Recently, down-regulation of miR-145 by DNA methylation and p53 mutation pathways has been suggested in prostate cancer [24]. We searched a Web site (microRNA.org-Targets and Expression; http://microrna.org/microrna) for specific targets of miR-145 and retrieved more than 20 targets with high matching scores. However, no association has been suggested between these candidate targets and ATL in the literature. Potential targets of miR-145 have been reported including MYC in colon cancer [25], ERG in prostate cancer [26], and connective tissue growth factor in glioblastoma [27].

In conclusion, we investigated ATL cell lines and clinical ATL cases for miRNA expression using miRNA arrays and quantitative RT-PCR, and we found that down-regulation of miR-145 was highly associated with a worsened clinical course in patients. An in vitro functional assay showed that miR-145 expression was inversely associated with tumor cell proliferation. To the best of our knowledge, the involvement of miR-145 in ATL has not been reported. Our findings shed light on the biological and clinical roles of miR-145 in ATL and provide the basis for the development of new miRNA-targeted therapeutic strategies against this tumor.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2014.01.017.

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