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Effects of expressing human T-cell leukemia virus type 1 (HTLV-I) oncoprotein Tax on *DOK1*, *DOK2* and *DOK3* gene expression in mice

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ABSTRACT. Transgenic mice expressing the *tax* gene from human T-cell leukemia virus type 1 (HTLV-I) genome developed T-cell leukemia or histiocytic sarcoma after at least 12 months. The transgenic mice showed low expression of the downstream of tyrosine kinase (DOK) family members, *DOK1*, *DOK2* and *DOK3*, which were recently reported to be tumor suppressor genes. Mice showed low *DOK2* expression at 5–6 months of age, before disease onset. The expression of *DOK1* and *DOK3* was not significantly reduced at any age tested. These results suggest that downregulation of *DOK2* by the expression of the viral *tax* gene is the first step in the development of T-cell leukemia or histiocytic sarcoma.

KEY WORDS: adult T-cell leukemia/lymphoma (ATLL), downstream of tyrosine kinase (DOK), human T-cell leukemia virus type 1 (HTLV-1)

Human T-cell leukemia virus type 1 (HTLV-I) was the first human retrovirus to be isolated. Infection with HTLV-I can result in the aggressive malignancy adult T-cell leukemia/lymphoma (ATLL) or in inflammatory diseases, such as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and HTLV-I uveitis, after a prolonged period of latency, often lasting 20–50 years [15, 16]. HTLV-I encodes the oncoprotein Tax, which modulates the expression of several genes leading to T cell transformation and appears to be a key molecule in the development of ATLL. Tax also interferes with the functions of several tumor suppressor proteins [3, 7, 15, 16]. To investigate the pathogenic role of Tax *in vivo*, my research group created a transgenic (Tg) mouse model of HTLV-I using the distal promoter of *Lck* to express *tax* in mature thymocytes and peripheral T lymphocytes [10]. Over 2 years, 28.1% of the Tax-expressing Tg (Tax-Tg) mice developed mature T cell leukemia/lymphoma compared with only 1% of their non-Tg littermates [9]. A few aged Tax-Tg mice developed HAM/TSP-like disease with symmetrical paraparesis of the hind limbs, which was caused by the infiltration of bone-marrow-derived histiocytic sarcoma (HS) cells [12].

The downstream of tyrosine kinase (DOK) family of proteins has seven members, DOK1–DOK7, which are adaptor proteins that modulate tyrosine kinase signaling [5]. DOK1, DOK2 and DOK3 are preferentially expressed in hematopoietic cells, and DOK1 and DOK2 inhibit BCR-ABL-driven leukemogenesis in mice [8, 17]. *DOK*-knockout mice were recently used to show that DOK1, DOK2 and DOK3 contribute to tumor suppression in lung tumor and aggressive HS [1, 6]. We have also reported that *DOK2* and *DOK3* expression is significantly reduced in HTLV-I-infected cell lines *in vitro* [11]. In this study, we investigated the expression of the *DOK1*, *DOK2* and *DOK3* genes in Tax-Tg mice with leukemia or HS (HAM/TSP-like disease) and the relationship between the expression of the *tax* and *DOK* genes *in vivo*.

The generation of Tg mice expressing the *tax* gene under the control of the distal *Lck* promoter is described elsewhere [9, 10]. The Tax-Tg mice and the littermate control mice were maintained under specific pathogen-free conditions in laminar-flow benches at 22 ± 2°C with a 12 hr light/dark cycle. Food (CE-2; CLEA, Tokyo, Japan) and water were supplied *ad libitum*. Over a 2-year period, the Tax-Tg mice spontaneously developed T-cell leukemia with an incidence of approximately 20% and HS with an incidence of only 5%. T-cell leukemia and HS were evaluated based on the pathological findings and a flow-cytometric analysis of the mouse splenocytes (Fig. 1b). Four of the mice with T-cell leukemia mice and three of mice with HS were used in this study. This study was carried out in strict accordance with the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan. All procedures involving animals and their care were approved by the Animal Care Committee of Kumamoto University in accordance with the regulations for animal experiments outlined by Kumamoto University. Single-cell suspensions were prepared from the spleens of healthy Tax-Tg mice, those with T cell leukemia/lymphoma or HS (HAM/TSP-like), and their non-Tg littermates, which were used as controls. The splenic cell populations

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were examined with flow cytometry [9, 10, 12]. The spleen samples were treated with ammonium chloride to lyse the red blood cells. The cells were then stained with fluorescein isothiocyanate-conjugated anti-CD45 (30-F11; eBioscience, San Diego, CA, U.S.A.), anti-CD3 (145-2C11; eBioscience), anti-CD19 (MB19-1; eBioscience), anti-CD11b (M1/70; eBioscience), anti-Ly-6G (Gr-1; RB6-8C5; eBioscience), anti-Mac-2 (M3/38; BioLegend, San Diego, CA, U.S.A.), anti-CD68 (FA-11, BioLegend) or anti-F4/80 antibody (BM8, BioLegend). Flow cytometry was performed with a FACSCalibur (Becton Dickinson; Franklin Lakes, NJ, U.S.A.) or Guava easyCyte 6HT/2L flow cytometer (Merck Millipore, Billerica, MA, U.S.A.) with FlowJo software (Tree Star, Ashland, OR, U.S.A.). Total RNA was isolated from the spleens of the T cell leukemia/lymphoma mice, HS (HAM/TSP-like) mice, age-matched healthy Tax-Tg mice and non-Tg mice using the RNeasy Mini, RNA isolation kit (Qiagen, Valencia, CA, U.S.A.). Approximately 0.2 μg of total RNA was reverse transcribed with the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). Real-time PCR was used to measure *DOK1*, *DOK2*, *DOK3* and HTLV-1 *tax* expression on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) or a Roche LightCycler 480 System II (Roche Diagnostics, Indianapolis, IN, U.S.A.). The primers used to amplify the *tax* region of HTLV-I are described elsewhere [13]. The murine *DOK1*, *DOK2* and *DOK3* primers used were: *DOK1* sense, 5′-TCCACCTGAGACAGTTGCTG-3′ and antisense, 5′-TGGTCATCCCTTGACTCCTC-3′; *DOK2* sense, 5′-ACCATACGACCCACAGAAGC-3′ and antisense, 5′-GAAACCTGTAGGGCCAGTCA-3′; and *DOK3* sense, 5′-TCCAACTGAGGGAGACATCC-3′ and antisense, 5′-CAGCCTCAAACGAGAACACA-3′. The real-time PCR cycling parameters were: 1 min at 95°C, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. The expression levels of the target genes were normalized to the expression of the murine gene encoding β-actin (*ACTB*). The primers for *ACTB* were: *ACTB* sense, 5′-TGTCCCTGTATGCCTCTGGT-3′ and antisense, 5′-GATGTCACGCACGATTTCC-3′.

The expression levels of *tax*, *DOK1*, *DOK2* and *DOK3* were analyzed by real-time reverse transcription (RT)–PCR in the T cell leukemia/lymphoma mice, HS (HAM/TSP-like) mice, age-matched healthy Tax-Tg mice (the same litters) and non-Tg mice. The expression levels of *tax* in the spleens of the healthy and leukemic mice were not significantly different, whereas the *tax* expression in the Tax-Tg mice with HS was low (Fig. 1a). This may be due to the increased proportion of HS cells ($CD3^{-}CD19^{-}$) in the splenocytes (>50%) and the decreased number of Tax-expressing T cells ($CD3^{+}$) in HS mice, whereas $CD3^{+}$ leukemic cells were most frequent (>80%) in mice with leukemia (Fig. 1b). These results are consistent with my previous reports [9, 10, 12]. The relative mRNA expression of the *DOK* genes in the spleens of the non-Tg mice, age-matched healthy Tax-Tg mice, leukemic mice and HS mice is shown in Fig. 1c. The relative expression of *DOK1*, *DOK2* and *DOK3* was significantly lower in the Tax-Tg mice with leukemia or HS than in the non-Tg mice. It has been reported that mice lacking *DOK* genes succumb to chronic myelogenous leukemia or aggressive HS [6, 8, 17]. These reports suggest that the expression of the *DOK* genes inhibited the onset of hematopoietic and lymphoid tumors, including leukemia/lymphoma and HS, in mice. Therefore, the *DOK* genes might also be associated with the onset of T-cell leukemia and HS caused by Tax of HTLV-I.

The Tax-Tg mice showed that the expression of Tax significantly reduced *DOK2* expression before the development of any disease, compared with that in the non-Tg mice (Fig. 1c). We investigated the changes in *DOK* gene expression in the healthy Tax-Tg at 5–13 months of age before disease onset (Fig. 2). *DOK2* expression was already low in the Tax-Tg mice at 5–6 months of age, and expression of *DOK2* remained constantly low after this time. These results suggest that downregulation of *DOK2* expression is the first step in the development of disease characterized by low expression of *DOK* genes. By contrast, the expression of *DOK1* and *DOK3* was not significantly reduced at 5–13 months (Fig. 2). *DOK1*, *DOK2* and *DOK3* are preferentially expressed in immune cells. *DOK1* mRNA is expressed in B and T cells, in myeloid cells, such as macrophages and neutrophils, and in $CD4^{-}CD8^{-}$ thymocytes and pro-B ($B220^{+}CD43^{+}IgM$) lymphocytes. Similarly, *DOK2* mRNA is preferentially expressed in T cells and myeloid cells, with little or no expression in B cells. By contrast, *DOK3* mRNA is preferentially expressed in myeloid cells and B cells, with little or no expression in T cells [5]. The Tax-Tg mice, in which *tax* was expressed from the distal *Lck* promoter, express Tax in mature thymocytes and peripheral T cells [9, 10], so Tax might first inhibit the expression of the *DOK2* gene in $CD3^{+}$ T cells. Furthermore, *Lck* is also expressed in a subset of B cells known as ‘B-1’ cells [2, 4, 14], so the expression of *DOK1* and *DOK3* might be slightly reduced in Tax-Tg mice.

It has been reported that the heterozygous loss of *DOK2* can promote tumorigenesis. Moreover, DOK1, DOK2 and DOK3 have overlapping functions and can cooperate in tumor suppression [1, 6, 8, 17]. Further studies are required to evaluate whether DOK1 and DOK3 are key factors in the development of leukemia and HS in Tax-Tg mice or ATLL patients.

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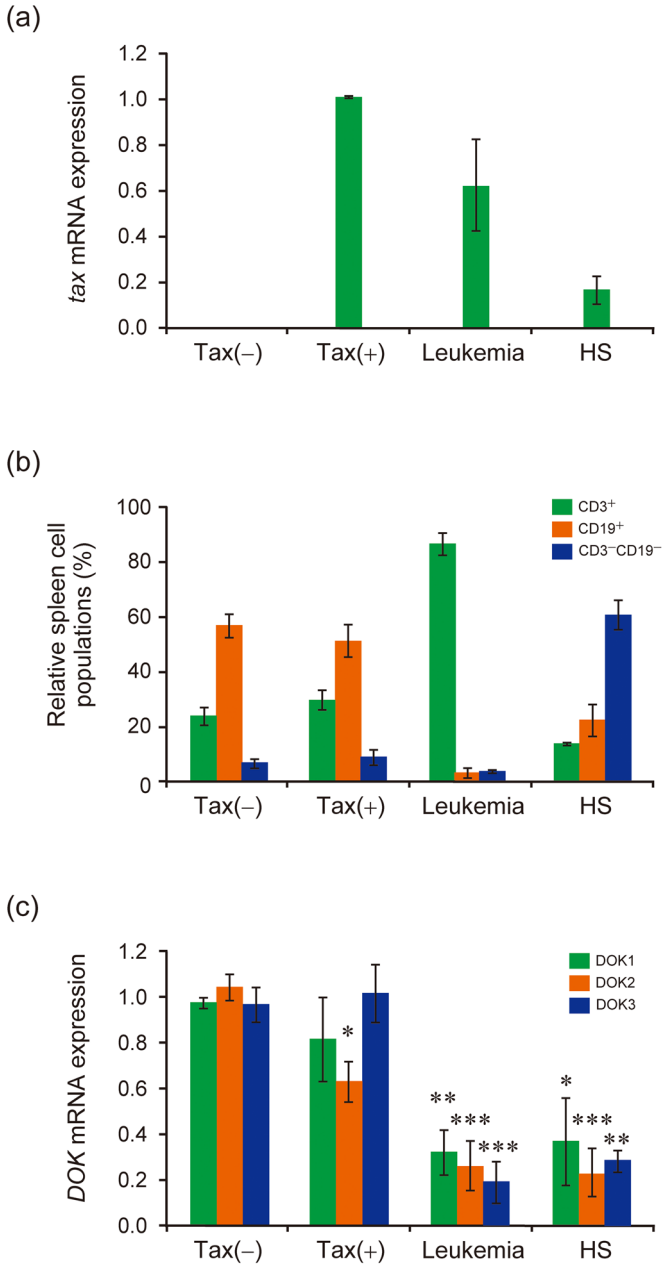

Fig. 1. Reduced *DOK* gene expression in Tax-Tg mice with leukemia or HS. (a) Relative mRNA expression of *tax* in the splenocytes of non-Tg (Tax[−]) mice (n=5), healthy Tax-Tg (Tax[+]) (n=4) mice, Tax-Tg mice with T cell leukemia (leukemia) (n=4) and Tax-Tg mice with histiocytic sarcoma (HS) (n=3). The expression of *tax* in the spleens of the healthy Tax-Tg mice was arbitrarily set to 1.0. (b) Relative spleen cell populations in Tax (−) (n=5), Tax (+) (n=4), leukemia (n=4) and HS mice (n=3) were assessed with flow cytometry. (c) Relative mRNA expression of *DOK1*, *DOK2* and *DOK3* in the spleens of Tax (−) (n=5), Tax (+) (n=4), leukemia (n=4) and HS mice (n=3). The expression of each *DOK* gene in the spleens of Tax (−) mice was arbitrarily set to 1.0. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed with Dunnett's test. *P<0.05, **P<0.01 and ***P<0.001 vs non-Tg mice.

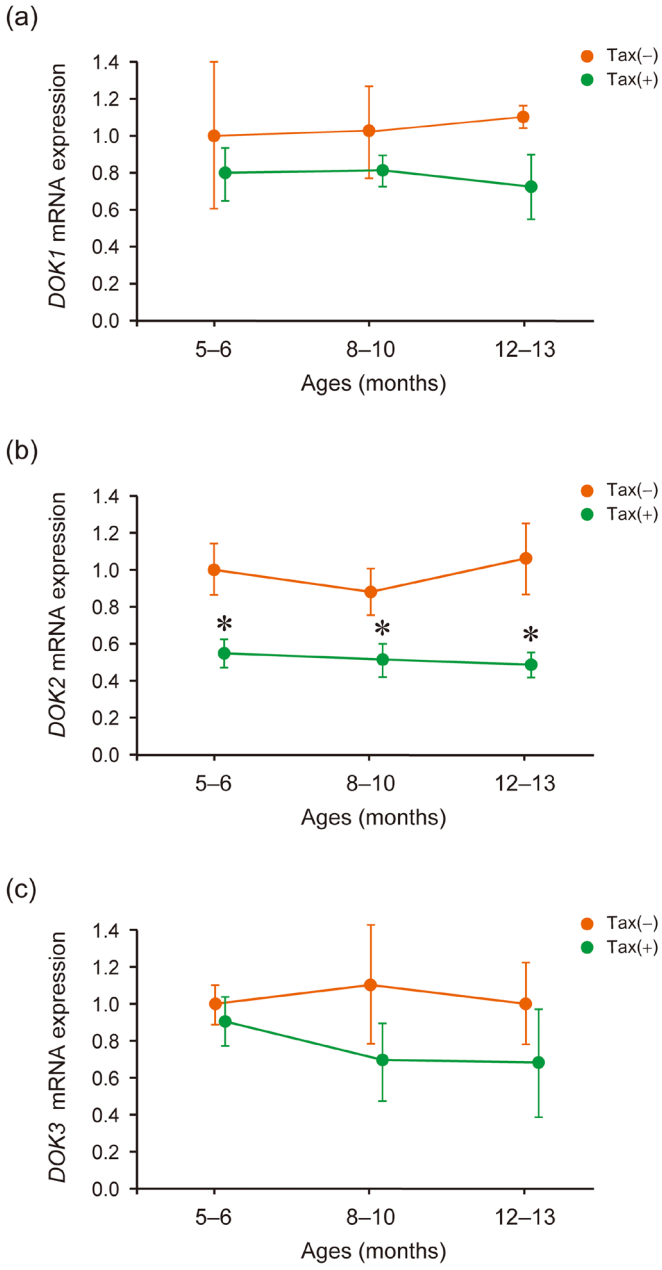

Fig. 2. *DOK2* gene expression is reduced in Tax-Tg mice before disease onset. (a) *DOK1* expression in non-Tg (Tax[−]) and healthy Tax-Tg (Tax[+]) mice between 5 and 13 months of age (n=5 per group). (b) *DOK2* expression in Tax (−) and Tax (+) mice between 5 and 13 months of age (n=5 per group). (c) *DOK3* expression in Tax (−) and Ta (+) mice between 5 and 13 months of age (n=5 per group). Data are presented as the mean ± standard error of the mean. Statistical analysis was performed with Student's t-test. *P<0.05 vs non-Tg mice.

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