Identification of the transgenic integration site in 2C T cell receptor transgenic mice

Chae-Yeon Son · Brian B. Haines · Andreas Luch · Chun Jeih Ryu

Received: 16 April 2018 / Accepted: 14 August 2018 / Published online: 21 August 2018 © Springer Nature Switzerland AG 2018

Abstract

2C T cell receptor (TCR) transgenic mice have been long used to study the molecular basis of TCR binding to peptide/major compatibility complexes and the cytotoxicity mechanism of cytotoxic T lymphocytes (CTLs). To study the role of variable gene promoters in allelic exclusion, we previously constructed mutant mice in which the Vβ13 promoter was deleted (P13 mice). Introduction of 2C transgene into P13 mice accelerated the onset of systemic CD8 T cell lymphoma between 14 and 27 weeks of age, although parental P13 mice appeared to be normal. This observation suggests that the lymphoma development may be linked to features of 2C transgene. To identify the integration site of 2C transgene, Southern blotting identified a 2C-specific DNA fragment by 3′ region probe of 2C TCR α transgene, and digestion-circularization-polymerase chain reaction (DC-PCR) amplified the 2C-specific DNA fragment with inverse primers specific to the southern probe. Sequence analysis revealed that DC-PCR product contained the probe sequences and the junction sequences of integration site, indicating that 2C TCR α transgene is integrated into chromosome 1. Further genomic analysis revealed cytosolic phospholipase A2 group IVA (cPLA2) as the nearest gene to the integration site. cPLA2 expression was upregulated in the normal thymi and T cell lymphomas from 2C transgenic mice.

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s11248-018-0090-1) contains supplementary material, which is available to authorized users.

Chae-Yeon Son and Brian B. Haines have contributed equally to this work.

C.-Y. Son · C. J. Ryu
Department of Integrative Bioscience and Biotechnology, Institute of Anticancer Medicine Development, Sejong University, Seoul, Korea
E-mail: cjryu@sejong.ac.kr

B. B. Haines · A. Luch
Koch Institute for Integrative Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA

Present Address:
A. Luch
Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Present Address:
B. B. Haines
Merck Research Laboratory, 33 Avenue Louis Pasteur, Boston, MA 02115, USA
although it was not altered in the lymph nodes of 2C transgenic mice. The result is the first report demonstrating the integration site of 2C TCR transgene, and will facilitate the proper use of 2C transgenic mice in studies of CTLs.

**Keywords**
- 2C TCR transgenic mice
- T cell lymphoma
- Integration site
- DC-PCR
- cPLA2

**Abbreviations**
- TCR: T cell receptor
- pMHC: Peptide/major compatibility complex
- DC-PCR: Digestion-circularization polymer chain reaction
- cPLA2: Cytosolic phospholipase A2 group IVA
- CTL: Cytotoxic T lymphocyte
- P13: Vβ13 promoter deficient mice
- ZBED4: Zinc finger domain containing 4
- PBMC: Peripheral blood mononuclear cell
- FITC: Fluorescein isothiocyanate
- PE: Phycoerythin

**Introduction**

The 2C T cell receptor (TCR) clone was generated to study the molecular basis of TCR binding to peptide/MHC (pMHC) complexes (Kranz et al. 1984). The 2C clone is a CD8⁺ mouse T cell clone (H-2b) that expresses a MHC-I-restricted αβ TCR (Kranz et al. 1984). 2C transgenic mice were also generated to investigate the cytotoxicity mechanism of cytotoxic T lymphocytes (CTLs) of a given specificity (Sha et al. 1988). 2C transgenic mice (type A) have approximately 2 copies of β transgene and 4 copies of α transgene (Sha et al. 1988). In addition to a fully rearranged TCR Vβ8.2-Dβ2-Jβ2.4 gene, 2C β construct has germline TCR Vβ5.2, Vβ8.3, Vβ5.1, and Vβ14 genes additionally (Sha et al. 1988). 2C α construct contains a productive Vα3.1-Jα rearrangement and additional joining segment genes (Sha et al. 1988). 2C transgenic mice express the same αβ antigen receptor from the CTL clone 2C, and the 2C TCR is expressed on 20–95% of peripheral blood T lymphocytes in the transgenic mice (Saito et al. 1984; Sha et al. 1988). The 2C TCR clones from the transgenic mice are able to lyse targets with the same specificity as the original 2C clone (Saito et al. 1984; Sha et al. 1988). Multiple pMHC ligands to 2C TCR have been identified. 2C TCR interacts with an endogenous Kᵇ MHC-I when it is bound to dEV8 (EQYKFYSV) (Tallquist et al. 1996). 2C TCR interacts with the alloantigen Lᵃ when it is bound to the synthetic peptide QL9 (QLSPFPFDL) (Sykulev et al. 1994). 2C TCR also recognizes Kᵇ when it is bound to SIY (SIYRYYGL) (Udaka et al. 1996). Furthermore, there are high-resolution crystal structures of both 2C TCR and 2C TCR-pMHC complex (Colf et al. 2007; Degano et al. 2000; Garcia et al. 1996). Thus, 2C transgenic mice have been extensively utilized over 30 years to understand the molecular biology of CTLs.

A previous study reported that all 2C transgenic mice develop T cell lymphomas when they are crossed to Tpl2 proto-oncogene-deficient mice, although parental mouse strains (Tpl2⁻/⁻ and 2C transgenic mice) show no signs of T cell malignancy (Tsatsanis et al. 2008). Most of the tumor cells are 2C CD8 single positive (SP) T cells in 2CTpl2⁻/⁻ mice, suggesting that 2C TCR-positive T cells turn into CD8 SP T cell lymphomas when the cells are chronically stimulated in Tpl2⁻/⁻ background. The results suggest the possibility that the lymphoma development may be linked to features of 2C transgene. Previously, we constructed mutant mice in which a 1.2-kb region of the Vβ13 promoter was either deleted (P13⁻/⁻) or replaced with the simian virus 40 minimal promoter plus five copies of Gal4 DNA sequences (P13⁻R/R) (Ryu et al. 2004). When 2C TCR transgenic mice were crossed to both P13⁻/⁻ and P13⁻R/R mice (both called P13 mice), in this study, we also found that all 2CP13 mice spontaneously developed systemic T cell lymphomas while parental P13 mice appeared to be normal. The result also suggests that 2C TCR transgenic mice are susceptible to the T cell lymphoma development. However, the integration site of 2C TCR transgene has not been reported until now. Here, we report for the first time the integration site of 2C TCR transgene, which is probably associated with the onset of systemic CD8 T cell lymphoma.
Materials and methods

Mice and genotyping

2C and F5 TCR transgenic mice were described previously (Mamalaki et al. 1993; Sha et al. 1988). P13\(^{-/-}\) and P13\(^{R/R}\) mice were also described previously (Ryu et al. 2004). The 2C transgenic RAG1\(^{-/-}\) mice (2CRAG\(^{-/-}\)) (Manning et al. 1997) were backcrossed to the C57BL/6 (B6, H-2\(^{b}\)) background, and 2CP13\(^{+/-}\) mice were primarily generated by mating 2CRAG1\(^{+/-}\) mice with P13\(^{-/-}\) mice. Studies were carried out according to institutional guidelines for animal use and care. Mice were genotyped for 2C expression by a flow cytometric assay of peripheral blood mononuclear cells (PBMCs) with the clonotypic antibody 1B2. PCR genotyping assays for P13 mice were performed as described previously (Maurice et al. 2001; Ryu et al. 2004). The PCR conditions were performed as follows: 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 3 min, and final extension at 72 °C for 10 min. To identify 2C transgene junction in 2C transgenic mice, 6R primer (5\'-GCTCATCAACTAAACACTGAA-C-3\') and 2C-333/2C-333-nested primers (5\'-ATA-CATCCGGTGAACTGCT-3\') were used for 2C genotyping PCR. As a control, β-actin gene was also amplified by PCR with two primers (5\'-GACATCCGGTAAAGACCTC-TAT-3' and 5\'-TTGATCTCTCATGCTCAGA-3').

Cell culture

Two thymic lymphoma cell lines P101 and P99 were derived from p53\(^{-/-}\) mice and cultured in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum and antibiotics (Haines et al. 2006). Other thymic lymphoma cells were derived from 2CP13\(^{+/-}\) (P13) and 2CP13\(^{+/-}\) (R18) mice and were grown in the same medium. The medium was replaced every 3 days.

Southern blot analysis

To detect the 2C TCR-specific DNA fragment, 2C TCR genomic DNAs were analyzed by Southern blot analysis. Genomic DNAs from wild type and 2C transgenic mice were digested with EcoRi or BamHI, fractionated, and transferred to Nylon membrane (Biorad, Seoul, Korea). The subsequent fragments were detected by hybridization using 3' region probe of 2C TCR α construct (3'TCR α probe in Fig. S1). The 3'TCR α probe was obtained using the primers TSP68 (5'-CTCTGCACACTTGCCGCACT-3') and TSP67 (5'-TGACACCTGCGCTCAGCAT-3') in a PCR reaction. P\(^{32}\)-radiolabeled DNA probe was generated using the random primer DNA labeling kit (Roche, Seoul, Korea).

Digestion-circularization PCR (DC-PCR)

Genomic DNAs of 2CP13\(^{+/-}\) and P13\(^{+/-}\) mice were digested with EcoRI or BamHI (Takara, Shiga, Japan). The purified DNAs were then self-circularized using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) at 16 °C for 16 h. The circularized DNAs were heated at 65 °C for 20 min and purified by QIAEX II Extraction Kit (Qiagen, Hilden, Germany). The amplification was performed in a 50 μl reaction mixture containing 500 ng template DNAs, 17.5 nmole each deoxynucleotide triphosphates, 10 pmol 6F and 6R primers, 5 μl of 10 × ExTaq buffer containing 2 mM MgCl\(_2\), and 2.5 unit of ExTaq (Takara). The gradient PCR conditions included initial denaturation at 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 48–63 °C for 30 s and 72 °C for 5 min, 20 cycles of 94 °C for 30 s, 48–63 °C for 30 s and 72 °C for 5 min, and a final extension step at 72 °C for 7 min. The amplified products were fractionated and visualized by ethidium bromide staining. The PCR products were further confirmed by nested PCRs with nested primers 1F and 2F, specific to sequences internal to 6F primer. The final PCR products were purified from the gel for cloning. The primer sequences of 6F, 6R, 1F and 2F are 5'-GACATCCCGTAAAGACCTCATGATC-CAAGGACAATGT-3' and 5'-GCAGATGATC-CAAGGACAATGT-3', respectively.

Cloning and sequencing

DC-PCR Products were amplified by PCR with 6F-SalI and 6R-Xbal primers and cloned into the SalI and Xbal sites of pBluescript KS + vector. The primer sequences of 6F-SalI and 6R-Xbal are 5'-CCAGTC-GACCAAGTTAACTCTTTATGTGATTGG-3' and 5'-GTGTCTTAG-ACTCATCAACTAAACACTGAA-3'.
3′, respectively. Cloned products were sequenced commercially (Cosmo Genetech, Seoul, Korea). Alignment of the flanking sequence to the mouse genome was performed by the BLAT genome browser of University of California Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgBlat).

Semi-quantitative Reverse transcriptase-PCR (RT-PCR)

Total RNAs were isolated from the thymi and lymph nodes of 5–8 week-old-wildtype, P13−/−, 2C, and 2CP13+/− mice using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNAs were also isolated from lymphoma cells of 2CP13+/− and 2CP13+/R mice. One-Step RT-PCR premix kit (Intron, Seoul, Korea) was mixed with specific primers for cPLA2. The PCR conditions were performed as follows: one cycle at 45°C for 60 min and at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min, and a final extension step at 72°C for 10 min. β-actin was used as a loading control. The cPLA2 primers used were 5′-TGTTCAACA-GAGTTTTGG-3′ and 5′-ACAGAGCAACGA-GATGG-3′. The signal intensities of cPLA2 expression were analyzed using the Image J.

Flow cytometry

Flow cytometry was performed after the isolation of PBMCs from mouse tail vein. First, red blood cells were lysed by resuspending cell pellets with ammonium chloride buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) for 5 min at room temperature. The remaining cells were washed with ice-cold FACS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, 1% bovine serum albumin, 0.02% NaN3). One million cells were then resuspended in FACS buffer. The antibodies used were as follows: 1B2 (specific for 2C TCR), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a (BD Biosciences, Seoul, Korea), phycoerythrin (PE)-conjugated anti-mouse CD4 (BD Biosciences). Propidium iodide (PI)-negative cells were analyzed using a FACSCalibur cytometer (BD Biosciences).

Results

Southern blot analysis of 2C TCR genomic DNA

To study the role of variable gene promoters in allelic exclusion, previously, we constructed P13 mice where the Vβ13 promoter was deleted (Ryu et al. 2004). When 2C TCR transgenic mice were crossed to P13 mice, interestingly, all 2CP13 mice died from systemic CD8 T cell lymphoma between 14 and 27 weeks of age while parental 2C and P13 mice appeared to be normal (Fig. 1a). Most of lymphoma cells found in thymus and spleen were CD8 SP lymphocytes (Fig. 1b). To see whether accelerated lymphoma development in 2CP13 mice was due to some general effect of transgenic TCR expression, P13 mice were crossed with another transgenic TCR αβ mouse termed F5 (Mamalaki et al. 1993). Neither F5 nor F5P13+/− mice succumbed to disease over the course of 1 year (Fig. 1a). Furthermore, some 2C TCR transgenic mice themselves spontaneously developed T cell lymphomas when they were aged (Fig. 1a). The results suggest that 2C TCR transgenic mice are susceptible to the T cell lymphoma development. To detect the integration site of 2C TCR transgene, therefore, we did Southern blot analysis with wildtype and 2C TCR genomic DNAs. To generate a Southern probe specific to 2C TCR transgene, thymic DNA from 2C TCR transgenic mice was amplified by PCR with TSP67 and 68 primers matched to 3′ region of 2C TCRα construct (3′TCR α probe, Fig. S1). Genomic DNAs from wild type and 2C TCR transgenic mice were digested with the indicated restriction enzyme, and were hybridized with P32-radiolabeled 3′ TCR α probe. The 5.5 and 2.0 kb DNA fragments were only detected in EcoRI-digested 2C DNA lane (Fig. 2a). A 2.8 kb DNA fragment was also specifically detected in BamHI-digested 2C DNA lane, suggesting that the 5.5, 2.0 and 2.8 kb fragments may contain a part of 2C transgene and the flanking sequences of the integration sites.

PCR amplification of 2C transgene integration site

To determine whether the 2C-specific fragments contain 2C transgene junction, we conducted DC-PCR with two primers (6F and 6R) specific to the 2C-specific DNA fragment (Fig. S2a). Genomic DNAs were isolated from 2C+P13+/− (2C +) and
**Fig. 1** 2CP13 mice develop CD8 SP T cell lymphoma between 14 and 27 weeks of age. **a** Kaplan–Meier survival curves for various genotypes are expressed as percentage lymphoma-free survival as a function of time (weeks). Controls indicate mice including Wt, P13<sup>+/−</sup>, P13<sup>++</sup>, P13<sup>R/R</sup>, P13<sup>+/R</sup>, F5, F5P13<sup>+/−</sup> genotypes (n > 30 for all). Genotypes bearing lymphomas include 2C (n = 37), 2CP13<sup>+/−</sup> (n = 27), 2CP13<sup>+/+</sup> (n = 33), and 2CP13<sup>+/R</sup> (n = 38). **b** CD4 and CD8 cell surface phenotype of systemic lymphoma cells from a representative 15 week-old terminally ill 2CP13<sup>+/−</sup> mouse. Thymic and spleen origin of lymphoma cells were analyzed by flow cytometry with CD4 and CD8 antibodies.

**Fig. 2** Southern blot analysis of Wt and 2C thymic DNAs and amplification of 2C TCR integration site. **a** 2C and Wt genomic DNAs were digested with the indicated restriction enzymes, and transferred to Southern membrane after fractionation. 2C-specific fragments were detected by hybridization with 3′ TCR α probe. 2C-specific DNA fragments were indicated with arrowheads. **b** DC-PCR amplification of 2C transgene junction. Genomic DNAs of 2C<sup>+</sup> and 2C<sup>−</sup> mice were digested with EcoRI and self-ligated with T4 DNA ligase. DC-PCR was performed by 6F and 6R primers at various annealing temperatures. 2C-specific PCR products (2.0 kb) were indicated with arrows. The larger products were considered to be irrelevant because they were also present in the 2C<sup>−</sup> samples.
2C−P13+/− (2C −) mouse thymi and cleaved with EcoRI or BamHI. The DNAs were self-ligated and then subjected to DC-PCR with 6F and 6R primers. DC-PCR with BamHI-digested DNAs did not produce any PCR products. DC-PCR with EcoRI-digested DNAs did not produce 5.5 kb PCR products, either. However, DC-PCR with EcoRI-digested DNAs produced approximately 2 kb PCR products at 59.8 and 56.9 °C of annealing temperature in 2C − lanes but not in 2C −− lanes (Fig. 2b). To prove whether the 2 kb PCR product is 2C TCR-specific DNA fragment, the DC-PCR product was isolated and subjected to nested PCR with 1F and 2F primers (Fig. S2a). Nested PCRs also produced the expected DNA fragments (Fig. S2b). The result suggests that the amplified 2 kb fragment has 2C-specific DNA fragment and the flanking sequences of integration site.

Sequencing of 2C transgene junction reveals the integration site of 2C transgene

To identify the flanking sequence of 2C transgene, both the DC-PCR product and cloned DC-PCR product were subjected to sequence analysis. The results showed that both the primary DC-PCR product and the cloned DNA product have the same sequences (Fig. 3a). The full size of the DC-PCR product consisted of 1934 bp, and contained the fragment (535 bp) of 3′TCR α probe and unknown flanking sequences (1399 bp) (Fig. 3a). By using BLAT genome browsers (http://genome.ucsc.edu/cgi-bin/hgBlat), the unknown flanking sequences were aligned to mouse chromosome 1. Further analysis revealed that the 2C transgene was located between cytosolic phospholipase A2 group IVA (cPLA2) and Zinc finger domain containing 4 (ZBED4) at chromosome 1 (Fig. 3b).

Confirmation of 2C transgene junction

2C transgenic mice generally have CD4−CD8+ T lymphocytes in the peripheral blood (Sha et al. 1988). PBMCs were isolated from the tail blood of 2C and wildtype mice with various genotypes, such as 2CWt, Wt, 2CP13+/+, and 2CP13+/−. As expected, flow cytometric analysis with anti-CD4 and CD8 antibodies showed that 2C-positive mice only had CD8 SP lymphocytes in the PBMCs (Fig. S3a). Based on the sequence information of 2C transgene junction, we designed 2C-333 and 2C-333-nested primers complementary to the flanking sequences in chromosome 1 (Fig. 3a). When these primers were paired with 6R specific to 2C transgene, 333 bp-sized PCR product was expected. Therefore, genomic DNAs were isolated from the tail tips of 2C-positive and negative pups in the same litter, and subjected to PCR genotyping with transgene junction-specific primers. 2C-transgene junction-specific 333 bp bands were amplified by PCR with 6R and 2C-333 primers from 2C transgenic DNAs, whereas the transgene junction DNAs were not amplified from wildtype DNAs (Fig. S3b). The results confirmed the integration site of 2C transgene in chromosome 1.

Expression of the nearest cPLA2 gene

To examine whether 2C transgene integration affects the expression of the nearest gene cPLA2 of 2C TCR α transgene, total RNAs were isolated from thymic lymphoma cells of 2CP13+/− (P13), 2CP13+/R (R18) and p53+/− (P101 and P99) mice, and the RNAs were subjected to semi-quantitative RT-PCR analysis. As compared with wildtype, cPLA2 expression was increased by approximately 25–28-fold in 2C-derived lymphoma cells (P13 and R18) while it was not detected in p53+/−-derived lymphoma cells (P101 and P99) (Fig. 4), suggesting that increased expression of cPLA2 is associated with 2C transgene integration. To further figure out whether 2C transgene integration affects lymphoma development, total RNAs were isolated from the normal thymi and lymph nodes of wildtype, P13+/−, 2C, and 2CP13+/− mice before lymphoma development. cPLA2 expression was just slightly increased by approximately 1.3–1.5-fold in the lymph nodes of 2C-positive mice (Fig. 4b left panels). However, cPLA2 expression was significantly increased by approximately 8.7–11.1-fold in the thymi of 2C-positive mice (Fig. 4b right panels). The results suggest the possibility that the integration of 2C transgene is associated with T cell lymphoma development through altered expression of cPLA2 in the thymi of 2C-positive mice.

Discussion

2C transgenic mice develop CD8 SP T cell lymphomas when they have Tpl2−/− background,
although parental mouse strains (Tlp2−/− and 2C transgenic mice) show no signs of T-cell malignancy (Tsatsanis et al. 2008). In this study, we also found that 2C transgenic mice developed CD8 SP T cell lymphomas when they were crossed to P13 mice, and both parental mice did not develop any lymphomas until 23 weeks of age (Fig. 1a,b). It seems that the acceleration of T cell lymphomagenesis in 2C−/−P13−/− and 2C+P13R/R mice is due to the presence of 2C transgene. In the previous study, we cloned and sequenced Vβ13 signal ends during V(D)J recombination (Ryu et al. 2004). Interestingly, we found that all Vβ13 DNA cleavage products (100%) in P13−/− and P13R/R mice were derived from aberrant cleavages in the presence of 2C transgene, whereas the aberrant cleavages were observed in a small fraction of wildtype (13%), P13−/− (18%) and P13 R/R mice (47%) in the absence of 2C transgene. Therefore, it is possible to speculate that a continuation of the aberrant cleavages is the origin of genome instability and ends up with the development of T cell lymphoma in 2C−/−P13−/− and 2C+P13R/R mice.

**Fig. 3** Sequence analysis of the integration site of 2C TCR α construct. a Sequence analysis of DC-PCR product. 2C TCR transgene is shown in black-colored letters with PCR primers used. The franking sequence of 2C TCR α is shown in red-colored letters with PCR primers used. b Schematic diagram of 2C-TCR α transgene in chromosome 1. Transgene junction is indicated with an arrow. Newly designed PCR primers to amplify the integration junction is also shown as three arrows. Alignment of the flanking sequence to the mouse genome was performed with the BLAT genome browser of University of California Santa Cruz (UCSC). (Color figure online)
In this study, we also found that some of 2C transgenic mice also spontaneously developed T cell lymphoma when they were aged (Fig. 1a), suggesting that 2C transgene-containing mice may be prone to the development of T cell lymphoid malignancies because of 2C transgene itself. To generate 2C transgenic mice, 2C\textsubscript{a} and 2C\textsubscript{b} constructs (approximately 40 kb, respectively) were digested with the same Not I restriction enzyme and injected into fertilized eggs simultaneously (Sha et al. 1988). Therefore, it is highly likely that both 2C\textsubscript{a} and 2C\textsubscript{b} constructs form a concatemer that contains multiple copies of the 2C\textsubscript{a} and 2C\textsubscript{b} constructs linked in series in the same chromosome. As expected, 2C transgenic mice used in this study (type A) have 2 copies of the \(\beta\) transgene and 4 copies of the \(\alpha\) transgene (Sha et al. 1988). In addition to a fully rearranged TCR V\(\beta\)8.2 gene, 2C\textsubscript{b} construct also has germline TCR V\(\beta\)5.2, V\(\beta\)8.3, V\(\beta\)5.1, and V\(\beta\)14 genes additionally (Sha et al. 1988). 2C\textsubscript{a} construct also has additional joining segment genes (Sha et al. 1988). Therefore, the multiple copies of the additional TCR segment genes in 2C transgenic mice may be exposed to DNA cleavages during V(D)J recombination, and the DNA cleavages may be not strictly controlled, suggesting that the uncontrolled DNA cleavages in 2C-positive P13 mice cause genome instability, and 2C-positive P13 mice end up with the development of T cell lymphomagenesis. Further investigation is necessary to find the cause of T cell lymphomagenesis in 2C-positive P13 mice.

To figure out why 2C transgene accelerates T cell lymphomagenesis in 2C-positive P13 mice, in this study, we tried to clone the integration site of 2C transgene. We were not able to clone the integration site of 2C\textsubscript{b} transgene. Instead, we were able to clone the integration site of 2C\textsubscript{a} transgene, which was integrated into chromosome 1 (Fig. 3). The location of 2C\textsubscript{b} transgene has not been confirmed in this study. However, there is a high possibility that both 2C\textsubscript{a} and 2C\textsubscript{b} transgenes are integrated into the same chromosome.
1 as a concatemer, because both constructs were digested with the same Not I and injected into fertilized eggs simultaneously (Sha et al. 1988). As 2C transgene has enhancer and promoters for TCR gene expression, one thinks that the integration of 2C transgene may affect the expression of near genes. Actually, cPLA2, the nearest gene of the integration site, was abundantly detected in T cell lymphoma cells and normal thymic cells from 2C-positive P13 mice, whereas it was not detected in p53−/− mice-derived T cell lymphoma cells (Fig. 4a). Interestingly, cPLA2 expression was also increased in the thymi of 2C-positive P13 mice before the development of T cell lymphomas, while it was not significantly increased in the lymph nodes of 2C-positive P13 mice (Fig. 4b). The results suggest that cPLA2 expression is induced by TCR α and β enhancers of 2C transgenes, which are preferentially active in immature thymocytes (Bouvier et al. 1996; Carvajal and Sen 2000; Krangel et al. 2000). Overexpression of cPLA2 has been identified in a variety of cancers including non-small cell lung cancer, cholangiosarcomas, esophageal cancers, and cancers of the colon, ovarian, and small intestine (Nakanishi and Rosenberg 2006; Yarla et al. 2016). cPLA2 specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond, releasing arachidonic acid (AA) and lysophosphatidic acid. AA is further modified into eicosanoids, which are categorized as anti-inflammatory and inflammatory mediators (Yarla et al. 2016). Thus, cPLA2 can plays a key role in pathophysiology of various cancers and inflammation. cPLA2 is also an important factor for the function of CTLs. cPLA2 is required for CTL-mediated immunopathology independently of their TCR via NKG2D and IL-15 (Tang et al. 2009, 2013), suggesting that altered expression of cPLA2 dysregulates activity of CTLs through induction of inflammation. Furthermore, Vβ13 promoter knockout in P13 mice shows increased levels (18–47%) of aberrant DNA cleavages, as compared with that (13%) of wildtype mice (Ryu et al. 2004). Vβ13 promoter knockout also depletes a Vβ13-positive T cell subset from T cell population in P13 mice (Ryu et al. 2004). Therefore, immature thymocytes may initially undergo programmed cell death because of AA release and genome instability in 2C-positive P13 mice. However, some malignant T cell clones may be derived from 2C TCR-positive T lymphocytes through 2C TCR-mediated chronic stimulation on the situation of continuous induction of inflammation and genome instability in 2C-positive P13 mice. Therefore, it is tempting to speculate that combination of 2C transgene and P13 mutation gives synergistic effects on T cell lymphomagenesis. However, it still needs more studies to understand the exact correlation between T cell lymphomagenesis and combination of 2C transgene and P13 mutation.

The present study also brings up another interesting point. A recent study reported that although B16.SIY melanoma cells are triggered to express Kb, they are not well recognized by primed 2C+/RAG2−/−CD8+ T lymphocytes (Blank et al. 2004). However, PD-1-depleted 2C+/RAG2−/− T lymphocytes show augmented cytokine production and cytolytic activity against the melanoma cells compared to wildtype 2C lymphocytes (Blank et al. 2004), suggesting that PD-1 expression and activity may be altered in 2C transgenic mice. As both the 2C integration site and PD-1 gene are located in the same chromosome 1, it is possible that 2C integration makes 2C CTLs sensitive to PD-1-associated cell death. Actually, annexin V binding were upregulated in 2C thymocytes as compared with wildtype thymocytes (data not shown). Therefore, it could be interesting to study the relationship between PD-1 and 2C TCR integration site during T cell lymphomagenesis in 2C transgenic mice.

Acknowledgements This study was supported by the National Research Foundation of Korea (2016R1A2B4008610 and 2016M3A9C6918220).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T, Gajewski TF (2004) PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 64:1140–1145
Bouvier G, Watrin F, Naspetti M, Verthuy C, Naquet P, Ferrier P (1996) Deletion of the mouse T-cell receptor beta gene enhancer blocks alphabeta T-cell development. Proc Natl Acad Sci USA 93:7787–7881

Carvajal IM, Sen R (2000) Functional analysis of the murine TCR beta-chain gene enhancer. J Immunol 164:6332–6339

Colf LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, Garcia KC (2007) How a single T cell receptor recognizes both self and foreign MHC. Cell 129:135–146

Degano M, Garcia KC, Apostolopoulos V, Rudolph MG, Teyton L, Wilson IA (2000) A functional hot spot for antigen recognition in a superantigen TCR/MHC complex. Immunity 12:251–261

Garcia KC, Degano M, Stanfield RL, Brunmark A, Jackson MR, Peterson PA, Teyton L, Wilson IA (1996) An alphabeta T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. Science 274:209–219

Haines BB, Ryu CJ, Chang S, Protopopov A, Luch A, Kang YH, Draganov DD, Fragoso MF, Paik SG, Hong HJ, DePinho RA, Chen J (2006) Block of T cell development in PS3-deficient mice accelerates development of lymphomas with characteristic RAG-dependent cytogenetic alterations. Cancer Cell 9:109–120

Kranz DM, Sherman DH, Sitkovsky MV, Pasternack MS, Eisen HN (1984) Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera. Proc Natl Acad Sci USA 81:573–577

Mamalaki C, Elliott J, Norton T, Yannoutsos N, Townsend AR, Chandler P, Kioussis D (1993) Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. Dev Immunol 3:159–174

Manning TC, Rund LA, Gruber MM, Fallarino F, Gajewski TF, Kranz DM (1997) Antigen recognition and allogeneic tumor rejection in CD8+ TCR transgenic/RAG(-/-) mice. J Immunol 159:4665–4675

Maurice MM, Gould DS, Carroll J, Vugmeyster Y, Ploegh HL (2001) Positive selection of an MHC class-I restricted TCR in the absence of classical MHC class I molecules. Proc Natl Acad Sci USA 98:7437–7442

Nakanishi M, Rosenberg DW (2006) Roles of cPLA2alpha and arachidonic acid in cancer. Biochim Biophys Acta 1761:1335–1343

Ryu CJ, Haines BB, Lee HR, Kang YH, Draganov DD, Lee M, Whitehurst CE, Hong HJ, Chen J (2004) The T-cell receptor beta variable gene promoter is required for efficient V beta rearrangement but not allelic exclusion. Mol Cell Biol 24:7015–7023

Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S (1984) A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. Nature 312:36–40

Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, Loh DY (1988) Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. Nature 335:271–274

Sykulev Y, Brunmark A, Tsmidse T, Kageyama S, Jackson M, Peterson PA, Eisen HN (1994) High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. Proc Natl Acad Sci USA 91:11487–11491

Tallquist MD, Yun TJ, Pease LR (1996) A single T cell receptor recognizes structurally distinct MHC/peptide complexes with high specificity. J Exp Med 184:1017–1026

Tang F, Chen Z, Ciszewski C, Setty M, Solus J, Tretiakova M, Ebert E, Han J, Lin A, Guandalini S, Groh V, Spies T, Green P, Jabri B (2009) Cytosolic PLA2 is required for CTL-mediated immunopathology of celiac disease via NKG2D and IL-15. J Exp Med 206:707–719

Tang F, Sally B, Ciszewski C, Abadie V, Curran SA, Groh V, Fitzgerald O, Winchester RJ, Jabri B (2013) Interleukin 15 primes natural killer cells to kill via NKG2D and cPLA2 and this pathway is active in psoriatic arthritis. PLoS ONE 8:e76292

Tsatsanis C, Vaporiadi K, Zacharioudaki V, Androulidaki A, Sykulev Y, Margioris AN, Tsichlis PN (2008) Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. Proc Natl Acad Sci USA 105:2987–2992

Udaka K, Wiesmuller KH, Kienle S, Jung G, Walden P (1996) Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. J Immunol 157:670–678

Yarla NS, Bishayee A, Vadilakonda L, Chintala R, Duddukuri GR, Reddanna P, Dowluur KS (2016) Phospholipase A2 isoforms as novel targets for prevention and treatment of inflammatory and oncologic diseases. Curr Drug Targets 17:1940–1962