Acta Medica et Biologica
Vol.55, No.2, 55-60, 2007

Bcl11b Heterozygous Mice are Susceptible to N-methyl-N-nitrosourea-induced Thymic Lymphomas

Riko Funada

Departments of Molecular Genetics, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan

Received January 12, 2007, accepted January 19, 2007

Summary. N-methyl-N-nitrosourea (MNU) is an alkylating agent that can modify the guanine base in DNA. The mode of mutation induction differs from that of radiation, which generates large mutations such as chromosomal deletion and translocation. Our previous studies showed that *Bcl11b* is a tumor suppressor gene and that *Bcl11b*$^{+/-}$ heterozygous mice are susceptible to γ-ray induced thymic lymphomas. This study investigates whether or not the *Bcl11b*$^{+/-}$ genotype also provides susceptibility to MNU-induced thymic lymphomas. The results showed that loss of one copy of the *Bcl11b* gene contributed to lymphomagenesis, indicating that *Bcl11b* can suppress MNU induction of thymic lymphomas. Interestingly, loss of the wild-type *Bcl11b* allele was not detected in those lymphomas, contrasting with the finding of a high frequency of the loss in the γ-ray induced *Bcl11b*$^{+/-}$ thymic lymphomas. On the other hand, epigenetic inactivation of *Bcl11b* was observed in 74% (14/19) of the *Bcl11b*$^{+/-}$ lymphomas. This may be the main contributor to the loss of *Bcl11b* function and probably affects the elevation of lymphoma incidence and the shortening of the latency in *Bcl11b*$^{+/-}$ mice when MNU is administrated.

Key words— susceptibility to lymphomas, *Bcl11b*, N-methyl-N-nitrosourea, epigenetic inactivation.

INTRODUCTION

Mouse thymic lymphomas are one of the classic models of radiation-induced malignancies.[1-6] Our previous studies revealed allelic losses at a high frequency and mutations at *Bcl11b/Rit1*gene in γ-ray induced thymic lymphomas,[7] suggesting that *Bcl11b* is a tumor suppressor gene. *Bcl11b* (also called *CTIP2*) encodes zinc-finger transcription factors[8,9,10] that are homologous to a proto-oncogene, *Bcl11a/Evi9*.[11,12] *Bcl11b*$^{-/-}$ homozygous mice die soon after birth and exhibit developmental arrest at immature thymocyte stages and a decrease in the number of thymocytes to approximately one tenth of wild-type mice.[10] This decreased cellularity of thymocytes is ascribed in part to apoptosis in *Bcl11b*$^{-/-}$ thymocytes.[10,13] Since apoptosis has been considered as a mechanism to eliminate deleterious cells, the apoptotic phenotype of *Bcl11b*$^{-/-}$ thymocytes seems to contradict with the possibility of *Bcl11b* as a tumor suppressor. On the other hand, *Bcl11b*$^{+/-}$ heterozygous mice are viable and do not show phenotypic abnormalities.[14] However, when the heterozygous mice are irradiated at age of one month, they show a higher susceptibility to thymic lymphomas than wild-type mice, confirming the tumor suppressive property of *Bcl11b*.[15]

Administration of N-methyl-N-nitrosourea (MNU) to mice also develops thymyc lymphomas at a high efficiency.[16,17,18,19] MNU is an alkylating agent that can modify the guanine base in DNA; some of the modified guanines are changed to an adenine base after DNA replication. The mode of action differs from that of radiation; i.e., the major type of radiation-induced DNA changes is large mutations such as deletion and translocation. It is of interest, therefore, to examine whether or not the *Bcl11b*$^{+/-}$ genotype gives susceptibility to MNU-induced thymic lymphomas as well. The present study investigated DNA changes in thymic lymphomas induced by MNU using *Bcl11b*$^{+/-}$ heterozygous mice.

Correspondence: Riko Funada, Departments of Molecular Genetics, Graduate School of Medical and Dental Sciences, Niigata University, Asahimachi 1-757, Chuo-ku, Niigata 951-8510, Japan.

Abbreviations – MNU, N-methyl-N-nitrosourea; zfd, zinc finger domain.

MATERIALS AND METHODS

Mice and lymphoma induction

The *Bcl11b* knockout mice were originated in embryonic stem cell (ES cells) of the C57BL/6(B6) and DBA/2 F_1 genetic background, and the B6 allele of *Bcl11b* was disrupted by the insertion of a neo gene.[10] The *Bcl11b*$^{+/-}$ heterozygous mice of BALB/c(C) background were backcrossed more than six generations with BALB/c strain to obtain *Bcl11b*$^{+/-}$ mice of BALB/c background harboring the B6-derived chromosomal region in the vicinity of *Bcl11b* locus on mouse chromosome 12. The *Bcl11b*$^{+/-}$ mice were mated with BALB/c mice and their progeny were given a single intraperitoneal dose, 75 mg/Kg body weight, of MNU (Nakarai Co., Kyoto), when they aged six weeks. Development of thymic lymphoma was diagnosed by the inspection of labored breathing up to 250 days. Existence of tumors was confirmed upon autopsy of the mice.[7,16]

Genetic analysis

Isolation of genomic DNA from the brain and thymic lymphomas was carried out by standard protocols. Genotyping of *Bcl11b* was carried out with PCR as described previously.[7,12,13] Allelic loss at *Bcl11b* was analyzed by using *D12 Mit122* and *D12 Mit132* markers. The position of *D12 Mit122*, *Bcl11b*, and *D12 Mit132* was 102672267, 103384906, and 108207423, respectively, on chromosome 12 (from Ansambl data base). Analysis of *Bcl11b* mutations at the two zinc finger domain (zfd) regions was carried out with SSCP (single-stranded DNA conformational polymorphism) and subsequent direct sequencing (Fig.3). Sequences of the primers used are
5'-ACACCTCACCTGGGTGGTC-3'(zfd4F),
5'-CATTAGTCAGCAAGTGTTCACC-3'(zfd4R),
5'-TTTCCAGCTCTCTTCCCACGC-3'(zfd5F), and
5'-CCGAATTCTCTCTCAGCCTGCTCGATTTT-3'(zfd5R).
Analysis of K-*ras* mutations at the codons 12 and 13 was carried out with SSCP and subsequent direct sequencing. Sequences of the primers used are
5'-GCCTGCTGAAAATGACTGAG-3' and 5'-TCAAAG CGCACGCAACTGTG-3'.

Statistical analysis

χ^2 and P values for the difference between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{+/-}$ genotypes with the development of MNU-induced thymic lymphoma were obtained by the χ^2 test and Mantel-Cox test with StatView-J 5.0 software on a Machintosh personal computer.

Western blotting

Western blotting was performed as previously described.[10] Thymic lymphomas were suspended in PBS and mixed with an equal volume of lysis buffer, 0.125 M TrisHCl (pH 6.8), 10% Sucrose, 10% SDS 10% 2-ME and 0.004% bromophenol blue. The extract was electrophoresed in 8% SDS-PAGE gels and blotted onto Hybond membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein bands of *Bcl11bα* and *Bcl11bβ* were visualized using chemiluminescent detection (ECL plus, Amersham Pharmacia Biotech).

RESULTS

Bcl11b$^{+/-}$ genotype provides susceptibility to MNU-induced mouse thymic lymphomas

Bcl11b$^{+/-}$ BALB/c were mated with BALB/c mice and a total of 50 progeny were subjected to MNU administration. They were followed up for 250 days. Thirty-six of the mice developed tumors, 34 of which were thymic lymphomas. Genotyping of the *Bcl11b* locus revealed that 30 mice were of *Bcl11b*$^{+/-}$ and 27 thymic lymphomas had developed in them. Fig.1 displays the cumulative lymphoma incidence of mice of *Bcl11b*$^{+/-}$and *Bcl11b*$^{+/+}$ genotypes. *Bcl11b*$^{+/-}$ mice developed tumors at a higher incidence and a shorter latency than those of *Bcl11b*$^{+/+}$ ($P < 0.0001$ in Mantel-Cox test). This indicates that the *Bcl11b*$^{+/-}$ genotype provided susceptibility to MNU-induced thymic lymphomas, consistent with the fact that *Bcl11b* is a tumor suppressor gene.

Loss of *Bcl11b* expression in MNU-induced thymic lymphomas

Loss of the wild-type allele was examined using *D12Mit122* and *D12Mit132* markers in the 27 thymic lymphomas developed in *Bcl11b*$^{+/-}$ mice. These markers were able to distinguish BALB/c DNA from B6 DNA. Fig.2 shows examples of this analysis. *D12Mit122* and *D12Mit132* loci are centromeric and telomeric to the *Bcl11b* locus, respectively, and the C57BL/6 (B6) chromosomal region carries the *Bcl11b*-knockout allele whereas the BALB/c chromosome carries the wild-type allele. Only three of the 27 thymic lymphomas showed DNA alterations in the vicinity of *Bcl11b*, and none of the three lost the wild-type BALB/c allele. These result contrasted with the finding that loss of the wild-type allele were detected at a high frequency (54%) in γ-ray induced thymic lymphomas in *Bcl11b*$^{+/-}$ mice.[15]

Bcl11b mutations were examined at the two regions encoding zinc finger domains(zfd) in the 27 thymic

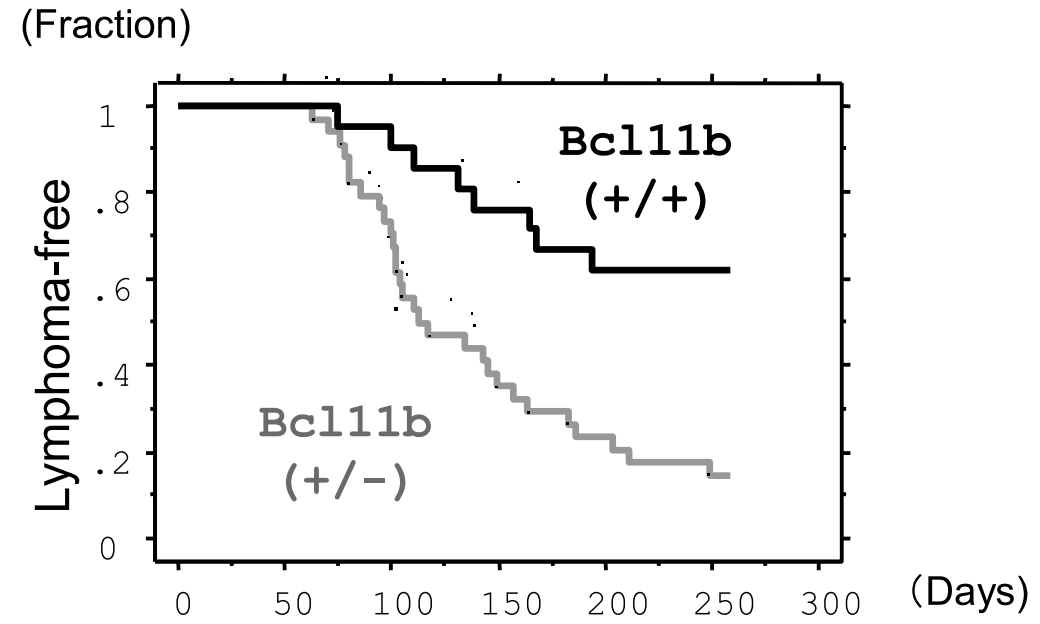

Fig. 1. Kaplan-Meier analysis of N-methyl-N-nitrosourea (MNU)-induced thymic lymphomas in *Bcl11b*$^{+/-}$ (*Gray line*) and *Bcl11b*$^{+/+}$ (*Black line*) mice. The x axis indicates time after MNU administration (days) and the y axis displays the fraction of mice free from lymphomas.

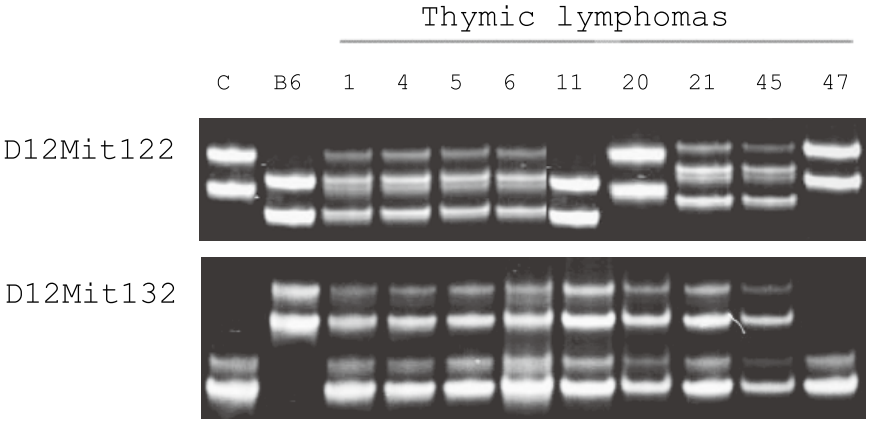

Fig. 2. Analysis of the loss of heterozygosity at the *Bcl11b* locus. The position of the *Bcl11b* locus is located between *D12Mit122* and *D12Mit132* on mouse chromosome 12. *C* and *B6* represent alleles of BALB/c and C57BL/6, respectively. The number of lymphomas is arbitrary. PCR products from one allele comprise two bands due to the nature of microsatellites. None of the lymphomas from *Bcl11b*$^{+/-}$ mice (including *lanes 21 and 45*) showed loss of the wild-type *Bcl11b* allele.

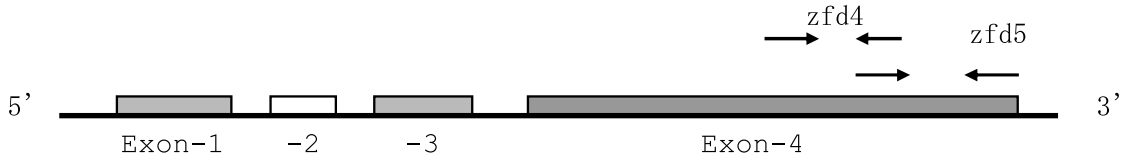

Fig. 3. Genomic structure of the *Bcl11b* gene. Four exons are shown as boxes on a bar. *Arrows* above the bar indicate the positions of PCR primers used for two zinc finger domain (zfd) regions (zfd4 and zfd5).

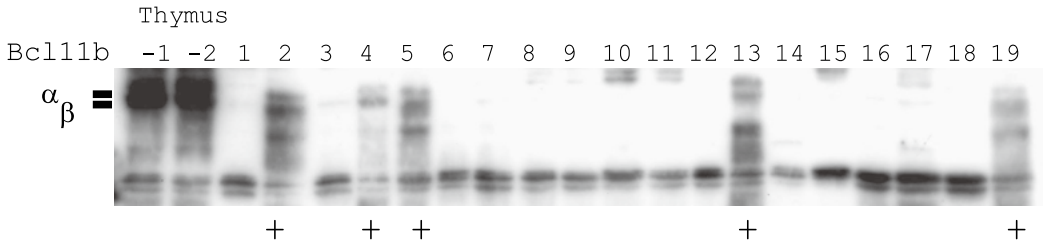

Fig. 4. Western blotting for the expression of *Bcl11b* proteins in of MNU-induced thymic lymphomas in *Bcl11b*$^{+/-}$ mice. *α* and *β* indicate the position of two isoforms, *Bcl11bα* and *Bcl11bβ*, respectively. Five lymphomas (*marked by* +) showed expressions, but the expression levels were lower than those in normal thymuses (indicated by thymus-1 and -2).

lymphomas (Fig.3). However, no DNA changes were detected (data not shown). Thus, we examined alterations of *Bcl11b* in the lymphomas at the protein expression level by Western blotting (Fig.4). Of the nineteenth lymphomas examined, only five expressed *Bcl11b* proteins. Furthermore, the levels of expression in the five were lower than that in the normal thymus. This high frequency in the loss of expression suggests the epigenetic inactivation of *Bcl11b* during lymphoma developments.

K-*ras* mutations

K-ras is much more frequently mutated in mouse thymic lymphomas than the other *H-ras* and *N-ras* genes, and the activation is attributed mostly to single point mutations localized to codon 12 or codon 13. Therefore, mutations of the codons 12 and 13 of *K-ras* gene were examined by single strand conformation (SSCP) and subsequent sequence analysis. The G to A substitution leading to the missense mutation from Glutamate to Aspartate was identified in eight of the MNU induced lymphomas, six being in the *Bcl11b*$^{+/-}$ lymphomas and two in the *Bcl11b*$^{+/+}$ lymphomas. The frequency (22%) of *K-ras* mutation in the *Bcl11b*$^{+/-}$ lymphomas was similar to that (approximately one fifth) in previous reports[7,6,19] and higher than that (approximately 5%) in *γ*-ray-induced lymphomas.[16,17,18,19]

DISCUSSION

The present study examined whether or not the *Bcl11b*$^{+/-}$ genotype provides susceptibility to MNU-induced thymic lymphomas. The results showed that loss of one copy of the *Bcl11b* gene contributes to lymphomagenesis. This indicates that *Bcl11b* functions as a tumor suppressor gene even in MNU induction of thymic lymphomas. Interestingly, loss of the wild-type *Bcl11b* allele was not detected in those lymphomas, contrasting with the finding in *γ*-ray induced thymic lymphomas in *Bcl11b*$^{+/-}$ mice that exhibited allelic losses at a high frequency (54%).[15]

Mutation analysis was performed for the zfd regions of *Bcl11b* and the codons 12 and 13 of *K-ras* gene. Although *K-ras* mutations were detected in the *Bcl11b*$^{+/-}$ lymphomas at similar frequencies to that in the *Bcl11b*$^{+/+}$ lymphomas (in this study, and references[16,17,18,19]), DNA changes were not detected in the zfd regions of *Bcl11b*. These might be due to differences in the mode of contribution to lymphomagenesis between *K-ras* and *Bcl11b*.

Epigenetic inactivation of *Bcl11b* was observed in 74% (14/19) of the *Bcl11b*$^{+/-}$ lymphomas, though its mechanism is unclear. This inactivation is probably the main contribution to the loss of *Bcl11b* function in the MNU-induced thymic lymphomas. When the inactivation occurred during the lymphoma development, the inactivation probably affects the elevation of lymphoma incidence and the shortening of the latency in *Bcl11b*$^{+/-}$ mice.

As described in the introduction, loss of the *Bcl11b* function in thymocytes leads to apoptosis at the developmental stage of CD4$^-$CD8$^-$CD44$^-$CD25$^-$ (the DN4 stage). At this stage thymocytes proliferate at a high rate. Therefore, the apoptosis given by the lack of the *Bcl11b* function eliminates proliferating cells from the thymus and hence seems to contradict the notion of *Bcl11b* as a tumor suppressor. However, the apoptosis might be a reflection of an elevated rate of proliferation, since hyperplastic or dysplastic cells in precancerous lesions often exhibit apoptosis and an accompanying high mitotic index.[20,21] Our previous study demonstrated that thymocytes in *Bcl11b*$^{+/-}$ mice exhibited loss of the wild-type allele and clonal growth at a certain precancerous stage after γ-irradiation.[15] The result suggests that those thymocytes well undergo cell proliferation and apoptosis at the same time through a balancing mechanism between cell division and cell death that is probably controlled by surrounding normal tissues.[22] Hence, it is possible that the epigenetic inactivation of *Bcl11b* provide a proliferative capability to thymocytes at a prelymphoma stage contributing to lymphomagenesis.

Acknowledgments. We thank Prof. R. Kominami and Prof. Y. Aoyagi for their expertise and helpful advice.

REFERENCES

1) Kaplan HS: The role of radiation in experimental leukemogenesis. *Natl Cancer Inst Monogr* **14**: 207-217, 1964.
2) Ludwig FC, Elashoff RM, Wellington JS: Murine radiation leukemia and the preleukemic state. *Lab Invest* **19**: 240-251, 1968.
3) Sado T, Kamisaku H, Kubo E: Bone marrow-thymus interactions during thymic lymphomagenesis induced by fractionated radiation exposure in B10 mice: analysis using bone marrow transplantation between Thy 1 congenic mice. *J Radiat Res* **32**: 168-180, 1991.
4) Kaplan HS, Carnes WH, Brown Brown MB, Hirsch BB: Indirect induction of lymphomas in irradiated mice. I. Tumor incidence and morphology in mice bearing nonirradiated thymic grafts. *Cancer Res* **16**: 422-425, 1956.
5) Humblet C, Defresne MP, Greimers R, Rongy AM, Boniver J: Further studies on the mechanism of radiation induced thymic lymphoma prevention by bone marrow transplantation in C57BL mice. *Leukemia* **3**: 813-818, 1989.
6) Kominami R, Niwa O: Radiation carcinogenesis in mouse thymic lymphomas. *Cancer Sci* **97:** 575-581, 2006.
7) Wakabayashi Y, Inoue J, Takahashi Y, Matsuki A, Kosugi-Okano H, Shinbo T, Mishima Y, Niwa O, Kominami R: Homozygous deletions and point mutations of the *Rit1/Bcl11b* gene in γ-ray induced mouse thymic lymphomas. *Biochem Biophy Res Comm* **301:** 598-603, 2003.
8) Avram D: Isolation of a novel family of C2H2 zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors. *J Biol Chem* **275:** 10315-10322, 2000.
9) Shinbo T, Matsuki A, Matsumoto Y, Kosugi S, Takahashi Y, Niwa O, Kominami R: Allelic loss mapping and physical delineation of a region harboring a putative thymic lymphoma suppressor gene on chromosome 12. *Oncogene* 18: 4131-4136, 1999.
10) Wakabayashi Y, Watanabe H, Inoue J, Takeda N, Sakata J, Mishima Y, Hitomi J, Yamamoto T, Utsuyama M, Niwa O, Aizawa S, Kominami R: *Bcl11b* is required for differentiation and survival of αβ T lymphocytes. *Nat Immunol* **4**: 533-539, 2003.
11) Nakamura T, Largaespada DA, Shaughnessy JD Jr, Jenkins NA, Copeland NG: Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. *Nat Genet* **12**: 149-153, 1996.
12) Liu P, Keller JR, Ortiz M, Tessarollo L, Rachel RA, Nakamura T, Jenkins NA, Copeland NG: *Bcl11a* is essential for normal lymphoid development. *Nat Immunol* **4**: 525, 2003.
13) Inoue J, Kanefuji T, Okazuka K, Watanabe H, Mishima Y, Kominami R: Expression of TCRαβ partly rescues developmental arrest and apoptosis of αβ T cells in *Bcl11b*$^{-/-}$ mice. *J Immunol* **176:** 5871-5879, 2006.
14) Kamimura K, Ohi H, Kubota T, Okazuka K, Yoshikai Y, Wakabayashi Y, Aoyagi Y, Mishima Y, Kominami R: Haploinsufficiency of *Bcl11b* for suppression of lymphomagenesis and thymocyte development. *Biochem Biophys Res Commun* (in press).
15) Ohi H, Mishima Y, Kamimura K, Maruyama M, Sasai K, Kominami R: Multi-step lymphomagenesis deduced from DNA changes in thymic lymphomas and atrophic thymuses at various times after γ-irradiation. *Oncogene* (in press).
16) Sato H, Tamura Y, Ochiai Y, Kodama Y, Hatakeyama

K, Niwa O, Kominami R: The *D4Mit12* locus on mouse chromosome 4 provides susceptibility to both γ-ray induced and N-methyl-N-nitrosourea-induced thymic lymphomas. *Cancer Sci* **94**: 668-671, 2003.
17) Newcomb EW, Steinberg JJ, Pellicer A: *ras* oncogenes and phenotypic staging in N-methylnitrosoures- and γ-irradiation-induced thymic lymphomas in C57BL/6J mice. *Cancer Res* 48: 5514-5521, 1988.
18) Diamond LE, Guerrero I, Pellicer A: Concomitant K-and N-ras gene point mutations in clonal murine lymphoma. *Mol Cell Biol* **8:** 2233-2236, 1988.
19) Shimada Y, Nishimura M, Kakinuma S, Takeuchi T, Ogiu T, Suzuki G, Nakata Y, Sasanuma S, Mita K, Sado T: Characteristic association between K-ras gene mutation with loss of heterozygosity in X-ray-induced thymic lymphomas of the B6C3F1 mouse. *Int J Radiat Biol* **77**: 465-473, 2001.
20) Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J: DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434:** 864-870, 2005.
21) Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr., Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD: Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**: 907-913, 2005.
22) Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* **100:** 57-70, 2000.