10% neutral buffered formaldehyde and representative slices were stained with hematoxylin–eosin (HE).

Western blot analysis. Proteins were extracted from tissues, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with appropriate antibodies as described previously.[4,8] The antibodies used in this study were: anti-ABL monoclonal antibody, Ab3 (Oncogene Science, Cambridge, MA, USA); an anti-Bcl11b polyclonal antibody;[8] and an antihistone H2AX antibody (Millipore, Bedford, MA, USA). Positive signals were detected with the enhanced chemiluminescence system.

Southern blot analysis and genomic PCR. For Southern blotting, DNA was digested with restriction enzymes, separated in an agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-dCTP-labeled *TCRβ* probe. Genomic PCR was carried out using the following primers as described previously:[11] P1 (5′-TGCAGCTTTCCGGGCGATGCCA-3′), P2 (5′-ACTTTCCCAG-AACCCCACGC-3′), and P3 (5′-CCTGCTTGCCGAATATCAT-GGTGG-3′) for *Bcl11b*; and P1 (5′-TCACATTGTTTCCTTCG-GTGTCAC-3′), P2 (5′-AAGTGTTGTGATTGGGAAGCGTAG-3′), P3 (5′-AGATCCCGTTGACTGAACACAGG-3′), P4 (5′-TTCAG-GTTTTGTTGTCGCGCCGTAG-3′), and P5 (5′-TCAGCTCTTT-CTGTGAGGGAGGTGG-3′) for *H2AX*.

Northern blot analysis and RT-PCR. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), separated in 1.2% formaldehyde gel, blotted to a nylon membrane, and hybridized with a ^{32}P-dCTP-labeled *H2AX* probe. RT-PCR was carried out using the following primers as described previously:[11] 5′-CGAGCTCA-GGAAAGTGTCCGAG-3′ and 5′-GGAAATTCATGAGCGGG-GACTG-3′ for *Bcl11b*; 5′-CCTTCTGGAAGACTTGGCCTTC-3′ and 5′-GAGGAAGATGTGCCTGTTACC-3′ for *H2AX*; and 5′-TTCAACACCCCAGCCATGTA-3′ and 5′-CTCAGGAGGAG-CAATGATCT-3′ for *β-actin*.

Flow cytometric analysis. Cells were stained with FITC- or phycoerythrin (PE)-conjugated anti-Thy-1.2, anti-B220, anti-Mac1, and anti-Gr1 monoclonal antibodies (Pharmingen, San Diego, CA, USA), as described previously.[5]

Chromosomal analysis. Chromosomes were prepared by means of standard culture procedures for tumor cells and treated with trypsin-Giemsa as described previously.[12]

Patient samples and normal bone marrow cells. Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University.[13] Diagnosis of CML CP or CML BC (myeloid or B-lymphoid lineage) was carried out based on morphological, cytogenetic, and immunophenotypic analyses. Normal bone marrow cells were obtained from a healthy volunteer.

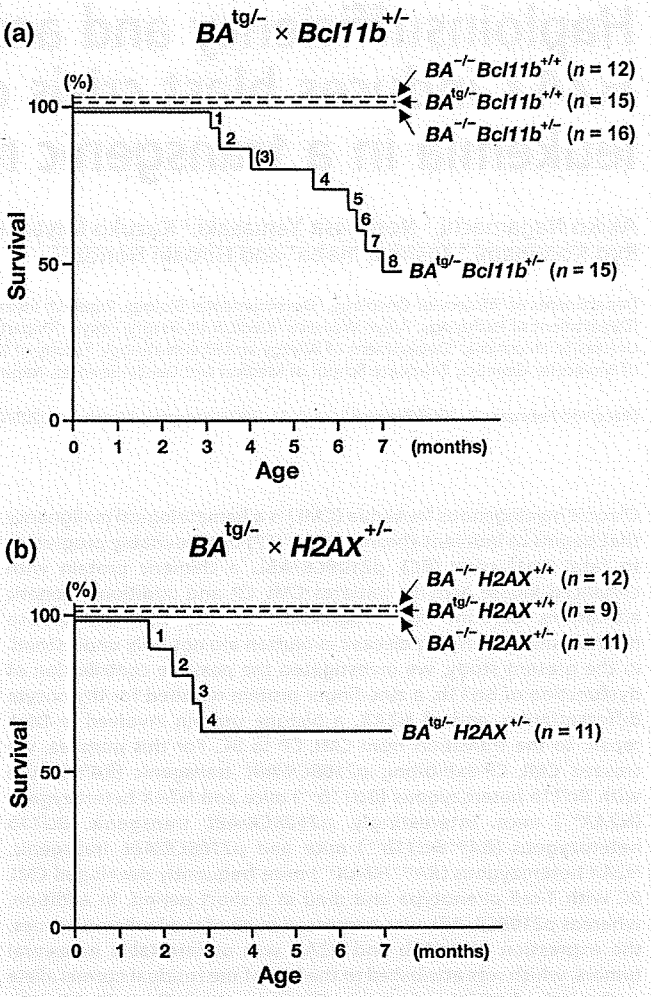

Fig. 1. Survival curves of mice generated by (a) $BA^{tg/-} \times Bcl11b^{+/-}$ and (b) $BA^{tg/-} \times H2AX^{+/-}$. The survival curves of $BA^{-/-}Bcl11b^{+/+}$ and $BA^{-/-}HX2A^{+/+}$, $BA^{tg/-}Bcl11b^{+/+}$ and $BA^{tg/-}H2AX^{+/+}$, $BA^{-/-}Bcl11b^{+/-}$ and $BA^{-/-}H2AX^{+/-}$, and $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ mice are shown as thin dotted, thick dotted, thin continuous, and thick continuous lines respectively. In the $BA^{tg/-} \times Bcl11b^{+/-}$ group, 8 of 15 $BA^{tg/-}Bcl11b^{+/-}$ mice died within 7 months of age and in the $BA^{tg/-} \times H2AX^{+/-}$ group, 4 of 11 $BA^{tg/-}H2AXb^{+/-}$ died within 3 months of age. The number of an unanalyzable $BA^{tg/-}Bcl11b^{+/-}$ mouse due to death (no. 3) is shown in parentheses.

Results

***$BA^{tg/-}Bcl11b^{+/-}$* and *$BA^{tg/-}H2AX^{+/-}$* mice developed acute leukemia and died in a short period.** To investigate the contribution of haploinsufficiency of *Bcl11b* and *H2AX* to the disease progression of CML, we crossed CML-exhibiting $BA^{tg/-}$ mice with $Bcl11b^{+/-}$ mice and $H2AX^{+/-}$ mice. Mice with four different genotypes were generated by each crossing: $BA^{tg/-} \times Bcl11b^{+/-}$ created $BA^{-/-}Bcl11b^{+/+}$ (wild type), $BA^{tg/-}Bcl11b^{+/+}$ (*p210BCR/ABL* transgenic), $BA^{-/-}Bcl11b^{+/-}$ (*Bcl11b* heterozygous), and $BA^{tg/-}Bcl11b^{+/-}$ (*p210BCR/ABL* transgenic, *Bcl11b* heterozygous); and $BA^{tg/-} \times H2AX^{+/-}$ produced $BA^{-/-}H2AX^{+/+}$ (wild type), $BA^{tg/-}H2AX^{+/+}$ (*p210BCR/ABL* transgenic), $BA^{-/-}H2AX^{+/-}$ (*H2AX* heterozygous), and $BA^{tg/-}H2AX^{+/-}$ (*p210BCR/ABL* transgenic, *H2AX* heterozygous). Mice with these genotypes were normally born approximately at the expected Mendelian ratio (see the mouse number shown in parentheses in Fig. 1), indicating that the crossing did not affect the embryonic development of the mice.

All of the mice were observed continuously and peripheral blood parameters were counted routinely. The genotype-based survival curves of the mice in each crossing are shown in Figure 1. During a 7-month observation period, in the $BA^{tg/-} \times Bcl11b^{+/-}$ group, 8 of 15 $BA^{tg/-}Bcl11b^{+/-}$ died of acute leukemia, in contrast $BA^{-/-}Bcl11b^{+/+}$, $BA^{tg/-}Bcl11b^{+/+}$, and $BA^{-/-}Bcl11b^{+/-}$ littermates did not show any disorders (Fig. 1a). As for the $BA^{tg/-} \times H2AX^{+/-}$ group (lower panel), 4 of 11 $BA^{tg/-}H2AX^{+/-}$ mice exhibited proliferation of blast cells and died within 3 months of birth, whereas no disease was observed in $BA^{-/-}H2AX^{+/+}$, $BA^{tg/-}H2AX^{+/+}$, and $BA^{-/-}H2AX^{+/-}$ littermates (Fig. 1b).

The representative results of pathological analysis of $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ leukemic mice are shown in Figure 2. Macroscopically, both leukemic mice exhibited marked thymic enlargement with splenomegaly, which were occasionally associated with lymph node swelling or pleural effusion (data not shown). The peripheral blood smears exhibited proliferation of blast cells morphologically resembling lymphoblasts (upper panels of Fig. 2). Tissue sections showed that the blast cells caused destruction of the basic structure of the thymus (second panels of Fig. 2) and infiltrated in non-hematopoietic tissues, such as liver (third panels of Fig. 2). In contrast, the bone marrow

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Table 1. Characteristics of *p210BCR/ABL*$^{tg/-}$ *Bcl11b*$^{+/-}$ leukemic mice

Mouse no.	Age at disease (months)	PB parameters			Macroscopic tumor sites	*TCRβ* status	p210BCR/ABL expression	Bcl11b expression	*Bcl11b* status
		WBC (× 10^3/μL)	Hb (g/dL)	Plt (× 10^4/μL)					
1	3.1	35.0	12.5	65.6	Thy, Spl	G/R	+	+	G/T
2	3.3	5.0	10.5	64.8	Thy, Spl	G/loss	+	+	G/T
3	4.0†	ND	ND	ND	Thy	ND	ND	ND	ND
4	5.3	2.3	7.1	44.2	Thy	G/loss	+	+	G/T
5	6.0	12.0	12.3	35.5	Thy, PE	G/loss	+	–	T/loss
6	6.1	6.6	13.9	53.5	Thy	G/R	+	–	T/loss
7	6.4	14.6	15.5	47.1	Thy, Spl	G/R	+	+	G/T
8	6.9	1.5	14.1	74.9	Thy, PE	G/R	+	–	T/loss

†Found dead. G, germline; Hb, hemoglobin; ND, not done; PB, peripheral blood; PE, pleural effusion; Plt, platelet; R, rearranged; Spl, spleen; T, targeted; Thy, thymus; WBC, white blood cell.

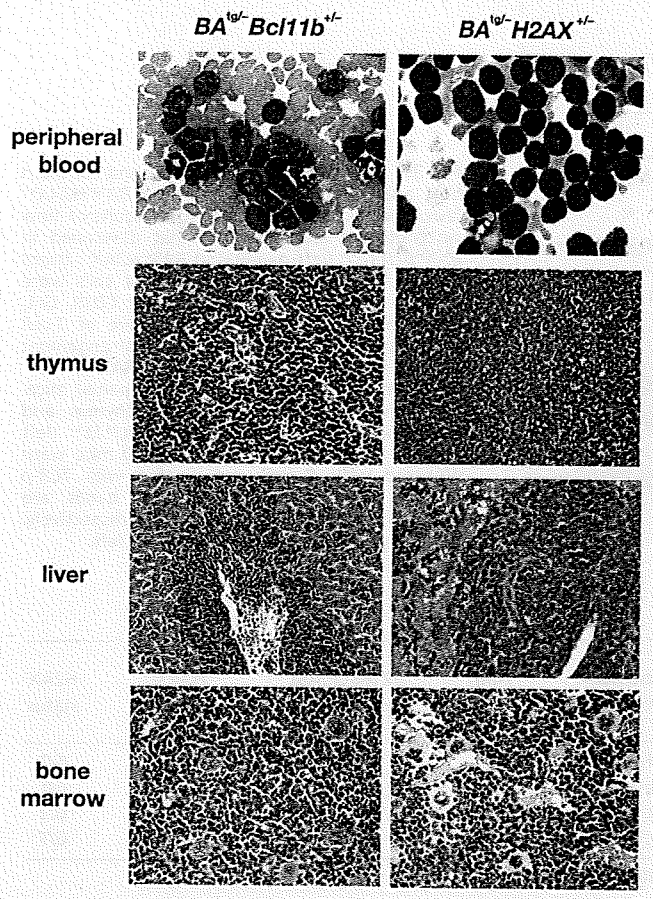

Fig. 2. Representative results of pathological analysis of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panels) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panels) leukemic mice. Wight-Giemsa-stained peripheral blood smears and HE-stained tissue slices are shown. In both leukemic mice, blast cells proliferated in the peripheral blood (upper panels), caused destruction of the basal structure of the thymus (second panels), and infiltrated around the vessel and in the sinusoids in the liver (third panels). In contrast, bone marrow exhibited myeloid cell hyperplasia with differentiation and proliferation of megakaryocytes (bottom panels).

showed a predominance of myeloid cells with differentiation and proliferation of megakaryocytes (bottom panels of Fig. 2). These results demonstrated that haploinsufficiency of *Bcl11b* and *H2AX* cooperated with *p210BCR/ABL*, transformed *p210BCR/ABL*-expressing hematopoietic cells, and caused CML BC. The characteristics of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice are summarized in Table 1 and Table 2, respectively.

Leukemias that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice were of T-cell lineage and were mostly clonal in origin. To determine the cell lineage and clonality of the leukemias that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice, blast cells were subjected to flow cytometric and Southern blot analyses.

The representative results of flow cytometric analysis of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic cells are shown in Figure 3(a). In both types of mice, leukemic cells were highly positive for Thy1.2, the antigen specific for T lymphocytes, but were negative for CD19, Gr1, and Mac1, the markers for B lymphocytes, granulocytes, and macrophages respectively.

The clonality of the leukemic cells was examined by gene rearrangement analysis. DNA extracted from a control thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were digested with a restriction enzyme and blotted with the T-cell receptor β (*TCR-β*) gene. As shown in Figure 3(b), more than half of the samples (no. 1 and no. 6–8 in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and no. 1 and 2 in *BA*$^{tg/-}$*H2AX*$^{+/-}$) showed rearranged bands, and in the remaining samples (no. 2, 4, and 5 in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and no. 3 and 4 in *BA*$^{tg/-}$*H2AX*$^{+/-}$), loss of the upper germline band was observed (the positions of germline bands are indicated by arrows and shown as 'G'). These results demonstrated that the blast cells of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were committed to the T-cell lineage and most of the tumors were clonal in origin.

Frequent and acquired loss of Bcl11b and H2AX protein expression in the tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice. We then investigated protein expression in the tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice. Proteins extracted from a control thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were blotted with antibodies against c-ABL, Bcl11b, and H2AX.

The results of p210BCR/ABL expression in these tumors are shown in the upper panels of Figure 4(a,b). As shown in both panels, the 210-kDa band was detected in all of the tumor samples, indicating that the blast cells originated from *p210BCR/ABL*-expressing hematopoietic precursors. We next examined the expression of Bcl11b and H2AX proteins in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic samples respectively. Interestingly, in the anti-Bcl11b western blot, the expression of Bcl11b was found to be lost in three of seven samples (no. 5, 6, and 8, middle panel of Fig. 4a). In addition, in the anti-H2AX blot, the expression of H2AX was undetectable in two of four samples (no. 2 and 3, middle panel of Fig. 4b). These results indicated that the protein expression of Bcl11b and H2AX was lost in several samples of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice.

To investigate the molecular mechanism underlying the loss of Bcl11b and H2AX expression, DNA extracted from tumor

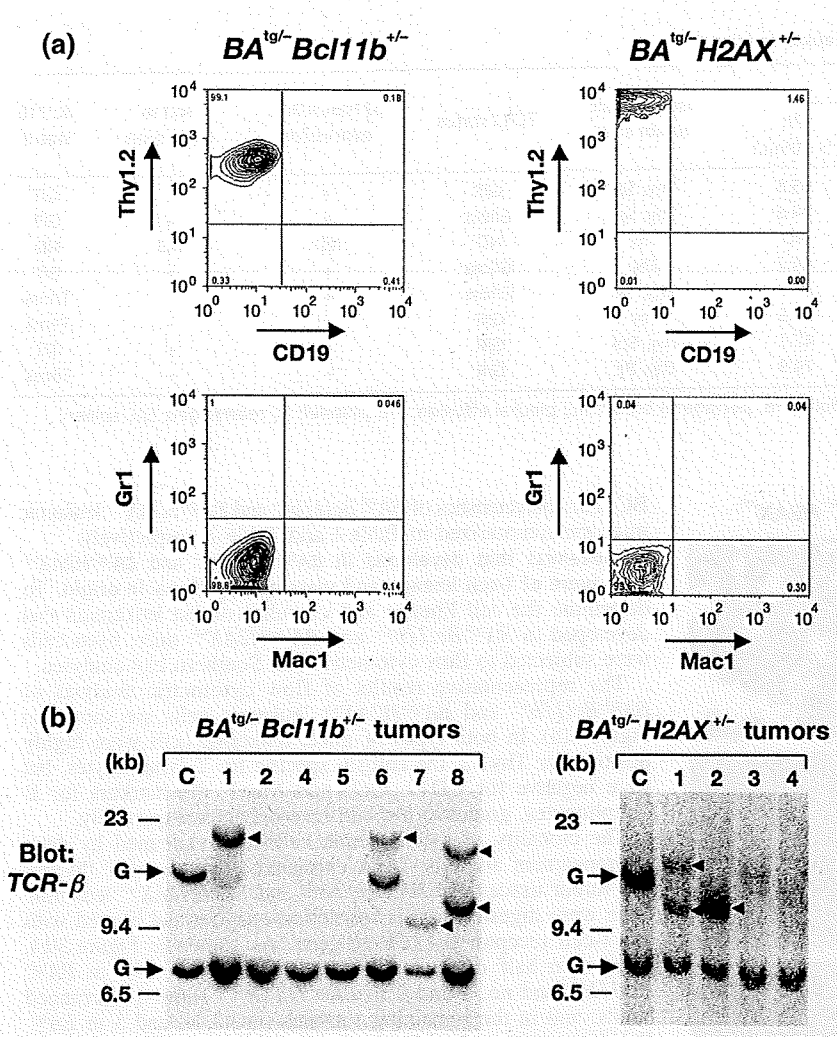

Fig. 3. Results of flow cytometric and Southern blot analyses of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice. (a) Representative results of flow cytometry of leukemic cells that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panel) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panel) mice. In both samples, blast cells were positive for Thy1.2 but negative for CD19, Gr1, and Mac1, indicating that they were of T-cell phenotype. (b) Results of gene rearrangement analysis in tumors that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panel) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panel) mice. (c) DNA extracted from control thymus and thymomas that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panel) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panel) mice were digested with *Bam*HI and blotted with *TCR-β* probe. Germline and rearranged bands are indicated by arrows and arrowheads respectively. Molecular markers are shown on the left.

Table 2. Characteristics of p210BCR/ABL$^{tg/-}$ H2AX$^{+/-}$ leukemic mice

Mouse no.	Age at disease (months)	PB parameters			Macroscopic tumor sites	*TCRβ* status	p210BCR/ABL expression	H2AX expression	H2AX status
		WBC (× 10³/μL)	Hb (g/dL)	Plt (× 10⁴/μL)					
1	1.8	15.3	16.1	56.4	Thy, Spl	G/R	+	+	G/T
2	2.2	160.8	10.4	53.4	Thy, Spl, LN	G/R	+	–	G/T
3	2.5	128.4	12.0	90.9	Thy, Spl, LN	G/loss	+	–	G/T
4	2.8	84.7	12.4	36.0	Thy, Spl, LN	G/loss	+	+	G/T

G, germline; LN, lymph node; R, rearranged; Spl, spleen; T, targeted; Thy, thymus.

tissues was subjected to genomic PCR that distinguished the PCR product of the wild-type allele from that of the knockout allele (upper panels of Fig. 4c,d). The results showed that the wild-type *Bcl11b* allele-derived band was not amplified in the three samples without Bcl11b expression (no. 5, 6, and 8, lower panel of Fig. 4c), indicating that the absence of Bcl11b protein was attributed to the loss of the residual wild-type *Bcl11b* allele. In contrast, the PCR product from the wild-type *H2AX* allele was retained in the two samples lacking H2AX expression (no. 2 and 3 in the lower panel of Fig. 4d). Because the PCR primer set detecting the wild-type allele (P1 + P2) did not amplify the coding region of the *H2AX* gene (upper panel of Fig. 4d), we designed another primer set encompassing the *H2AX* exon. As *H2AX* is a single-exon gene,[10] this primer set (shown as P4 and P5 in the upper panel of Fig. 4d) amplified a part of the promoter and the whole coding region. The results showed that a PCR product of expected size was detected in all of the *BA*$^{tg/-}$*H2AX*$^{+/-}$ tumors (lower panel of Fig. 4d). To examine the possibility that subtle deletion and/or base substitution had occurred in this region, we sequenced the whole PCR product but could not detect any mutation (data not shown). In addition, Southern blotting using a 5′ external probe for the *H2AX* gene[10] did not show any gross rearrangement (data not shown). These results indicated that the structure of the *H2AX* gene was largely unaffected. We next examined H2AX mRNA expression in the *BA*$^{tg/-}$*H2AX*$^{+/-}$ tumors by northern blotting. Interestingly, as shown

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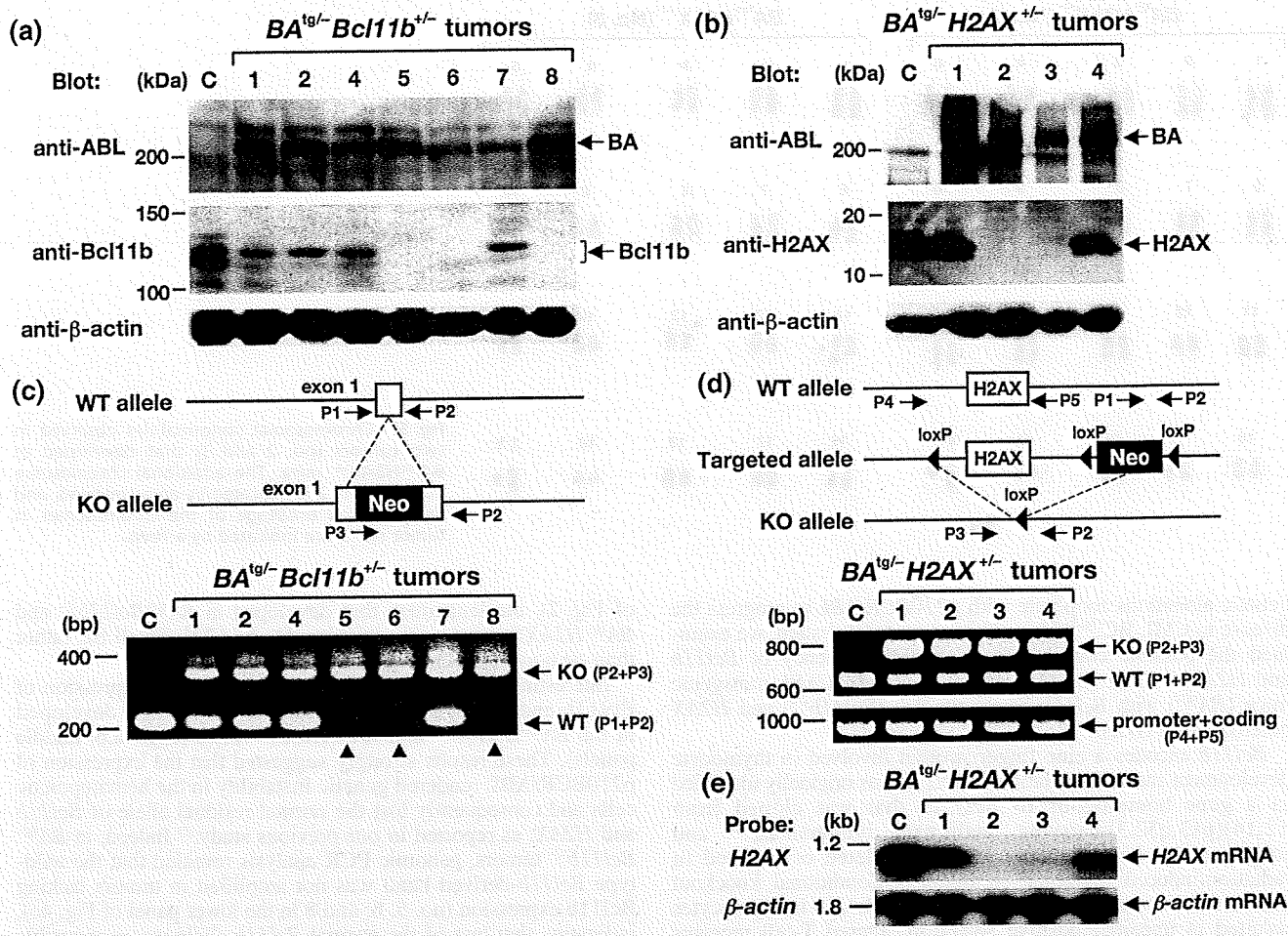

Fig. 4. Gene expression and PCR analyses of the tumors that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panels) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panels) mice. (a,b) Western blot analysis for the expression of p210BCR/ABL, Bcl11b, and H2AX proteins. Proteins extracted from a control (C) thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (no. 1, 2, and 4–8) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice (no. 1–4) were blotted with an anti-ABL antibody (upper panels) and anti-Bcl11b or anti-H2AX antibody (middle panels). The positions of p210BCR/ABL (BA), Bcl11b, and H2AX proteins are indicated by arrows. An anti-β-actin blot was carried out as an internal control (bottom panels). Protein markers are shown on the left. (c,d) Schematic illustrations of wild-type and targeted alleles for *Bcl11b* and *H2AX* genes (upper panels) and the resultant genomic PCR products (lower panels). DNA extracted from a control (C) thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (no. 1, 2 and 4–8) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice (no. 1–4) were amplified with sets of primers (P1 and P2 for wild-type [WT] alleles, P2 and P3 for knockout [KO] alleles, and P4 and P5 for a part of the promoter and the whole coding region of *H2AX*). The positions of primers are shown in the upper panels and WT- and KO-derived PCR products are indicated by arrows in the lower panels. Molecular markers are shown on the left. Samples without *Bcl11b* expression are indicated by arrowheads. *Neo*, neomycin resistance gene. (e) Expression of *H2AX* mRNA in *BA*$^{tg/-}$*H2AX*$^{+/-}$ tumors. RNA extracted from a control thymus (C) and tumor tissues of *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice (no. 1–4) were hybridized with *H2AX* cDNA probe. *β-Actin* hybridization was carried out as an internal control. Molecular markers are shown on the left.

in Figure 4(e), no H2AX mRNA was detected in tumors lacking H2AX protein expression (no. 2 and 3). These results indicated that the absence of H2AX protein was not due to deletion or mutation in the *H2AX* gene but to a lack of mRNA expression.

Chromosomal abnormalities in the leukemic cells developed in *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice. We finally examined the chromosomal status of the leukemic cells developed in *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice, as previous reports demonstrated that haploinsufficiency and absence of H2AX led to increased incidence of chromosomal abnormalities.[14,15] In the four tumors that arose from *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice, although two samples showed a normal karyotype (no. 1 and 4, data not shown), the other two samples (no. 2 and 3) that did not express H2AX protein (Fig. 4b) exhibited chromosomal aberrations. As shown in the left panel of Figure 5, sample no. 2 contained an additional chromosome (indicated by an arrowhead). In addition, as shown in the right panel of Figure 5, sample no. 3 exhibited deletions in the long arm of chromosome 6 and in the short arm of chromosome 13, and a breakage in chromosome 11 (indicated by arrows). These results suggested the possibility that the acquired loss of H2AX induced chromosomal instability and resulted in the chromosomal abnormalities observed in samples no. 2 and 3.

Discussion

Chronic myelogenous leukemia presents a paradigm for cancers that evolve through accumulation of genetic alterations. Generation of p210BCR/ABL initiates CML CP and additional genetic events progress the disease and develop CML BC.[1–3] Although chromosomal and molecular analyses revealed that various mechanisms are involved in the transition from CP to BC,[1–3] genes responsible for the evolution to BC have not fully been identified.

To elucidate the mechanisms underlying the disease evolution of CML, we have developed an *in vivo* model for CML in which expression of *p210BCR/ABL* induces CML CP, and additional

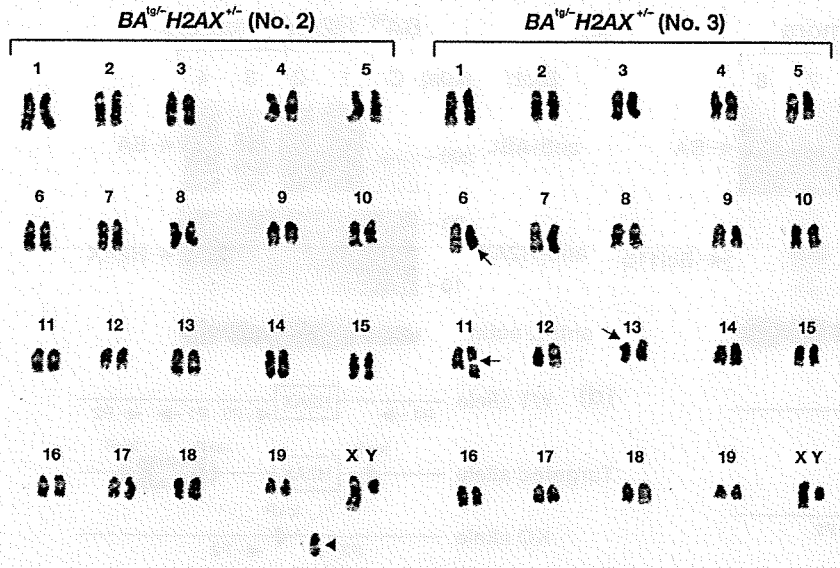

Fig. 5. Chromosomal abnormalities observed in two tumors (no. 2 and 3) that developed in *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice. The additional chromosome in tumor no. 2 is indicated by an arrowhead, and deletion and breakage of the chromosomes in tumor no. 3 are indicated by arrows.

genetic alterations cooperate with *p210BCR/ABL* to progress the disease to CML BC.[4–7] Using this as a model system, we examined the possible contribution of haploisufficiency of *Bcl11b* and *H2AX* to CML BC, by crossing *p210BCR/ABL* transgenic mice (*BA*$^{tg/-}$) with *Bcl11b* heterozygotes (*Bcl11b*$^{+/-}$) and *H2AX* heterozygotes (*H2AX*$^{+/-}$).

Bcl11b encodes a zinc finger protein involved in thymocyte development and differentiation.[8] *Bcl11b* was originally identified as a gene homologous to *Bcl11a*, that was cloned from t(2;14)(p13;q32.3)-carrying malignant lymphomas,[16] and subsequently shown to be frequently deleted or mutated in radiation-induced thymoma in mice.[17] Conditional knockout analysis showed that acquired ablation of *Bcl11b* in thymocytes resulted in impaired positive selection, altered T-cell receptor signaling, and reduced survival.[18] In addition, a recent study revealed that *Bcl11b* is involved in human leukemia carrying inv(14)(q11.2q32.31), which resulted in generation of the *Bcl11b–TRDC* fusion transcript.[19] On the other hand, H2AX is a member of the histone H2A family and a constituent of the nucleosome, the basic subunit of chromatin.[9,20,21] In response to the DNA double-strand break, H2AX rapidly becomes phosphorylated on the serine residue located at the C-terminus to form γH2AX at the DNA double-strand break sites.[9,20,21] This event creates a focus in the nucleus, where DNA repair and chromatin remodeling proteins are recruited.[9,20,21] In human hematopoietic malignancies, a single nucleotide polymorphism upstream of the *H2AX* gene was found to be tightly associated with susceptibility to non-Hodgkin lymphoma.[22] These results indicated that *Bcl11b* and *H2AX* are functionally implicated in cell differentiation and chromosomal stability, respectively, and are involved in subsets of hematopoietic malignancies.

We found that 8 of 15 *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ mice and 4 of 11 *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice developed acute leukemia and died in a short period (Fig. 1). These results indicated that haploinsufficiency of *Bcl11b* and *H2AX* conferred a growth advantage to *p210BCR/ABL*-expressing hematopoietic cells and consequently induced acute leukemia. The blast cells were highly malignant, as evidenced by massive proliferation in the peripheral blood, destruction of the basic structure of the thymus, and marked infiltration in non-hematopoietic tissues (upper 3 panels of Fig. 2). Surface marker analysis showed that the leukemic cells were of T-cell phenotype and Southern blot analysis demonstrated that most of the tumors were clonal in origin (Fig. 3). As the bone marrow showed the typical picture of CML CP (bottom panels of Fig. 2), the leukemias that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice were considered to be CML T-cell BC rather than *de novo* T-cell malignancy.

Interestingly, protein analysis revealed that the expression of Bcl11b and H2AX was lost in several tumors that developed in the *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice (Fig. 4a,b, middle panels). These results strongly suggested that the expression of p210BCR/ABL rendered genetic instability in the hematopoietic cells and consequently lost the normal residual allele of *Bcl11b* and *H2AX*, as reported in our previous study.[5] Indeed, in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ tumors, genomic PCR analysis revealed that the wild-type *Bcl11b*-derived band was not amplified in tumors lacking Bcl11b expression (no. 5, 6, and 8 in the lower panel of Fig. 4c), indicating that loss of the normal *Bcl11b* allele was responsible for the lack of the protein product. In contrast, the tumor tissues with no H2AX expression in *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice retained the normal *H2AX* allele, including the 3′ region, a part of the promoter region, and the whole coding region (no. 2 and 3 in the lower panels of Fig. 4d). Instead, we found that no H2AX mRNA was expressed in tumors lacking H2AX protein (no. 2 and 3 in the upper panel of Fig. 4e), which indicated that the absence of H2AX protein was due to the lack of H2AX mRNA expression. Although the mechanism underlying loss of the *H2AX* message in these tumors remains unclear, one possibility is that *p210BCR/ABL*-induced genetic alterations might have occurred in the other regions regulating *H2AX* transcription, such as the enhancer, which led to the loss of mRNA expression. Alternatively, p210BCR/ABL might have impaired the transcriptional machinery for H2AX mRNA in these tumors by an unknown mechanism. Taken together, our findings demonstrated that p210BCR/ABL induces loss of protein expression through several different mechanisms, including genomic instability and transcriptional inhibition.

It is to be noted that four *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and two *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice retained Bcl11b and H2AX protein expression (no. 1, 2, 4, and 7 in the middle panel of Fig. 4a and no. 1 and 4 in the middle panel of Fig. 4b). Thus, the mechanism of how haploinsufficiency of these genes caused disease evolution is to be clarified. Although no obvious phenotypic abnormalities were found in *Bcl11b*$^{+/-}$ or *H2AX*$^{+/-}$ mice, previous studies demonstrated that both types of heterozygotes exhibit enhanced susceptibility to hematological malignancies on *p53*$^{+/-}$ and *p53*$^{-/-}$ backgrounds.[14,15,23] These results indicated that both genes function as a dosage-dependent tumor suppressor and their

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haploinsufficiency predisposes to cancer development in certain genetic backgrounds. Thus, it is possible that haploinsufficiency of *Bcl11b* and *H2AX* exerted its oncogenic potential in cooperation with *p210BCR/ABL*, conferred a growth advantage to *p210BCR/ABL*-expressing hematopoietic cells, and consequently developed CML BC. An alternative possibility is that because p210BCR/ABL is known to promote genetic instability,[3,5] altered expression of unknown genes synergized with haploinsufficient *Bcl11b* or *H2AX* in *p210BCR/ABL*-expressing hematopoietic cells, accelerated progression of CML, and eventually caused CML BC.

We finally examined the possible chromosomal abnormalities in the leukemic cells of $BA^{tg/-}H2AX^{+/-}$ mice, as previous reports demonstrated that haploinsufficiency or deficiency of *H2AX* induced various chromosomal aberrations, especially on a $p53^{-/-}$ genetic background.[14,15] The results showed that two of four tumors exhibited chromosomal abnormalities, which were the presence of an additional chromosome, deletion in part of the long and short arms, and breakage in the body of several chromosomes (Fig. 5). Interestingly, $BA^{tg/-}H2AX^{+/-}$ mice with these chromosomal abnormalities exhibited very high white blood cell (WBC) counts ($>1 \times 10^5/\mu L$, see the right top panel of Fig. 2 and Table 2), suggesting that these events conferred a marked proliferative ability to *p210BCR/ABL*-expressing hematopoietic cells and exhibited a very aggressive phenotype. We also examined the possible contribution of dysfunction of genes involved in error-prone non-homologous end joining, such as *DNA ligase IV* and *XRCC4*, by crossing $BA^{tg/-}$ with *DNA ligase IV* heterozygous mice and *XRCC4* heterozygous mice. However, we did not observe disease acceleration or CML BC in $BA^{tg/-}$*DNA ligase* $IV^{+/-}$ or $BA^{tg/-}XRCC4^{+/-}$ double transgenic mice (data not shown), suggesting the possibility that among DNA repair-associated genes, *H2AX* might play a unique role in the disease evolution of CML.

The CML BC observed in $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ mice were of T-cell phenotype. Although T-cell BC is frequently observed in mouse models for CML,[5,24] it is rarely detected in human clinical samples. The reason for this discrepancy is not clear but one possibility is that human CML originates from the acquisition of *p210BCR/ABL*-transformed hematopoietic stem cells and the T-cell lineage is rarely involved probably due to its prolonged life span, whereas every cell in transgenic (or knockout) mice inherently contains (or lacks) the target gene and T cells might be more susceptible to the target gene-induced oncogenic transformation than other types of hematopoietic cells.

It is intriguing to examine whether acquired expressional loss of *Bcl11b* and *H2AX* contributes to human CML BC. We examined *Bcl11b* and *H2AX* expression in several CML BC samples by RT-PCR but did not detect the absence of mRNA expression in either gene (Supporting Information Fig. S1), probably due to the limited number of samples available and a lack of T-cell crisis cases. Thus, an expanded study is required to clarify the clinical significance of dysfunction of these genes in the development of CML BC.

In the present report, we demonstrated that haploinsufficiency and acquired loss of protein expression of *Bcl11b* and *H2AX* cooperate with *p210BCR/ABL* and induce CML BC. Our findings demonstrated that altered expression of genes involved in cell differentiation or chromosomal integrity contributes to the development of CML BC, which provides insights into the molecular mechanisms underlying the disease evolution of CML.

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Supporting information

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

Fig. S1. Expression of *Bcl11b* and *H2AX* in chronic myelogenous leukemia (CML) chronic phase (CP), CML blast crisis (BC), and normal bone marrow (BM). RNA extracted from one CML CP sample, four CML BC samples (two myeloid and two B-lymphoid), and one normal BM sample were subjected to RT-PCR for the expression of *Bcl11b* and *H2AX*. *β-Actin* RT-PCR was carried out as an internal control.

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Review Article

Preclinical Assays for Identifying Cancer Chemopreventive Phytochemicals

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Dietary factors influence carcinogenesis in a variety of tissues. The consumption of fruits and vegetables is associated with a decreased risk of several types of epithelial malignancies. In addition, there are interrelationships between diet, environmental factors, and genetics that can affect cancer risk. Potential chemopreventive agents against cancer development can be found among nutritive and/or nonnutritive compounds in inedible and edible plants. To identify potential cancer chemopreventive agents, scientists are evaluating hundreds of phytochemicals for the prevention of cancer. This short review article describes in vitro and in vivo assays reported to identify potential cancer preventive compounds from plants.

1. Introduction

Cancer mortality rates in the developed countries have increased throughout this century. It is already the leading cause of death in some Western countries [1, 2]. In Japan, the progressive introduction of Western dietary habits, especially increased fat intake and reduced carbohydrate and dietary fiber intake, has increased the incidence of colon cancer and related deaths [3]. Great advances have been made in the pharmacologic-based treatment of malignant epithelial neoplasms (cancers). In addition, there is a marked increase in the understanding of cell and molecular mechanisms underlying carcinogenic processes. However, therapy for advanced neoplastic disease remains limited. This may be due to the fact that advanced neoplasms contain a large number of genetic and molecular alterations that contribute to the maintenance of their neoplastic progression.

The chemopreventive approach against cancer development is highly attractive. Although highly attractive from a theoretical point of view, practical limitations may exist with respect to developing novel and effective chemopreventive agents. Most importantly, practical clinical endpoints are not definite. The efficacy of cancer chemoprevention can be determined by comparing the incidence of nondeveloped disease in a treated group to that of a control group. Such a clinical trial is labor intensive, very costly, and time-consuming. In addition, practically such trials cannot be conducted for the rapidly increasing number of chemopreventive agents being identified. These considerations suggest the use of certain intermediate biomarkers as indicators of clinical efficacy. Validated intermediate biomarkers thus may allow relatively small chemopreventive trials to be conducted within a short-term period. However, there are no universal approaches to determine intermediate biomarkers and their method of validation has still not been proven.

Some herbal and botanical products are likely to possess cancer preventive activities [4–7]. Many cancer patients use complementary and alternative medicines, including phytochemicals in addition to, or following the failure of standard cancer therapy [8]. The term phytochemical applies to any plant-based substance, but in the field of nutrition and cancer this term is usually applied to nutritive and nonnutritive chemicals that occur naturally in fruits and vegetables. A diet rich in fruits and vegetables has long been suggested to correlate with reduced risk of certain epithelial malignancies, including cancers in the lung, colon, prostate, oral cavity, and breast [4–7, 9–12]. Also, the cancer prevention potential of Mediterranean diets based mainly on

Table 1: Proposed mechanisms of phytochemicals for cancer prevention.

Food sources	Chemicals	Modification of carcinogen metabolism	Antioxidant and/or anti-inflammatory properties	Modification of cancer cell biology	Induction of differentiation	Antiangiogenesis	Apoptosis induction
Rosemary	Carnosol	+	+	+			+
	Rosmarinic acid	+	+	+		+	+
	Ursolic acid	+	+	+		+	+
Carrots	Carotenoids	+	+	+	+	+	+
Tumeric	Curcumin	+	+	+	+	+	+
Garlic	Diallyl sulfide	+	+	+	+	+	+
Gingko	Gingkolides	+	+	+			
Crucifers	Isothiocyanates	+	+	+	+	+	+
	Sulforaphane	+	+	+	+	+	+
Citrus	Limonene	+	+	+	+	+	+
Mint	Menthol	+	+	+	+		+
Cherries	Perillyl alcohol	+	+	+	+	+	+
Onion	Quercetin	+	+	+	+	+	+
Grape seed	Resveratrol	+	+	+	+	+	+
Milk thistle	Silymarin	+	+	+	+	+	+
Soybean	Isoflavones	+	+	+	+	+	+
Tea	Catechins	+	+	+	+	+	+
Olive	Oleuropein	+	+	+	+	+	+

olive tree products is known [13]. The major component of the leaves and unprocessed olive drupes of Olea europaea is oleuropein and the majority of polyphenols found in olive oil or table olives are derived from its hydrolysis. Oleuropein is a novel, naturally occurring antioxidant compound, which may possibly be used to prevent cancer [14–16] and cardiotoxicity induced by doxorubicin [17]. Searching for medicinal benefits from edible or inedible plants is not a new idea since numerous modern medicines have plant origins. Given that the ingestion of some plant foods results in reduced risk for cancer, researchers are delving into the identification of phytochemicals with cancer preventive ability in studies in vitro (cell culture), in vivo (model animals) and those in humans [18]. Phytochemicals can be roughly classified into four groups based on their mechanisms of chemopreventive action, as shown in Table 1. Preclinical studies focus on the identification of possible cancer preventive agents, short-term pharmacology, and assessment of toxicity. Agents that are within tolerable safety limits in humans then moved clinical trials to test their efficacy.

This review briefly summarizes the in vitro and in vivo assays used to discover possible new chemopreventive agents possessing novel mechanisms of action. Surely there are many more assay systems currently used in different laboratories to find candidate cancer chemopreventive agents and determine their efficacy. Because of limited space, only limited assays that might be useful for carcinogenesis and chemoprevention studies are herein introduced.

2. Anti-Inflammatory, Antioxidative, Antiangiogenic, and/or Apoptosis-Inducing Compounds in the Prevention of Cancer Development

One of the most exciting areas of cancer chemoprevention is the effect of anti-inflammatory compounds against CRC. Almost a dozen case-control and cohort studies have concluded that the daily or alternate day consumption of aspirin over extended periods results in halving one's risk for colorectal cancer (CRC) [19]. Several prospective studies are underway to specifically assess whether the daily consumption of aspirin affects the recurrence rates of colorectal adenoma in high-risk subjects for CRC. These studies strongly suggest that eicosanoids, especially prostaglandins (PGs), are involved in colorectal oncogenesis [20]. Two genes encode the two major forms of cyclooxygenase (COX) that is responsible for the metabolic conversion of dietary arachidonate to PGs, thromboxanes, and leukotrienes. The constitutive form, COX-1, is present in different types of cells. COX-2, the isoform inducible by growth factors and tumor promoters, is rarely found in the normal intestinal epithelial cells but is overexpressed in colorectal neoplasms [21–23]. Since COX-1 is a housekeeping gene, drugs for COX-1 inhibition cause side effects, such as ulceration. The kidney function, platelet aggregation, and the ulceration in stomach are strongly affected by the overuse or overdose of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) [24]. These limit the wide-scale application of NSAIDs in the general

public for the chemoprevention of CRC. Although NSAIDs are excellent chemopreventive agents in animal models for CRC, dietary aspirin does not inhibit azoxymethane (AOM)-induced colon carcinogenesis in rats that received a nonhigh fat diet. Because of side effects of NSAIDs that affect both COX-1 and COX-2 expressions, specific COX-2 inhibitors (-coxibs) were introduced to obtain cancer chemopreventive ability without side effects [25, 26]. They are indeed effective for CRC development in animal studies [27]. However, certain COX-2 inhibitors have been reported to increase the risk of ischemic heart diseases [28].

Many phytochemicals and/or botanicals are routinely tested for anti-inflammatory properties in the hope of finding new sources of medicines for the treatment of chronic inflammation and for the cancer chemoprevention. The plant world is apparently rich in sources of COX and/or inducible nitric oxide (iNOS) inhibitors that are involved in inflammation-related carcinogenesis [29]. Several of these have been found to inhibit cancer development in certain animal models. Curcumin [30] is able to inhibit the COX activity [31] in the skin [32] and suppresses skin cancer development in mice [33], tongue [34] and CRC in rats [35]. Tea [36, 37], given to human volunteers in a clinical trial, could reduce PGE_2 activity in the rectal mucosa [38]. Resveratrol [39] is a potent inhibitor of COX [40] and is an inhibitor in the mouse skin and rat mammary carcinogenesis models [41]. Silymarin [42] a COX and iNOS inhibitor [43] was also found to inhibit skin [44, 45], colon [46], tongue [47], and prostatic [45, 48] cancer development. However, the cancer preventive ability of carnosol and ginger substances, both COX inhibitors [49, 50], has not fully been investigated [51, 52]. Numerous phytochemicals thus remain to be tested in animal models for the most common cancers such as colon, mammary, prostate, and lung. Nobiletin [53–56], zerumbone [57, 58], and garcinol [59–61] are potent anti-inflammatory compounds present in plants that inhibit carcinogenesis in different tissues. A recent study demonstrated the chemopreventive ability of zerumbone, which has been shown to possess anti-inflammatory effects in mouse lung and colon carcinogenesis [62]. These phytochemicals [63], for example, curcumin [31, 64], resveratrol [65, 66], silymarin [67], from plants have multifunctional effects, including COX-2 inhibition and anti-inflammatory function, on a variety of events that involved carcinogenesis [68–70].

Most phytochemicals with chemopreventive ability [71] have direct antioxidant activity but many can also induce oxidative stress within cells when applied at high doses [11, 72]. For example, a simple phenolic acid protocatechuic acid is a potential cancer chemopreventive agent in a variety of tissues [73], but it enhances tumor promotion and oxidative stress in female ICR mouse skin via enhancing a promoter-induced inflammatory responses and promotion by affecting tyrosinase-dependent oxidative metabolism of protocatechuic acid [74]. Therefore, care should be paid for applying strong antioxidants to clinical use as a chemopreventive agent. However, recently anti-inflammatory, antioxidative chemopreventive phytochemicals targeting signal transduction mediated NF-2 related factor-2 (Nrf2), nuclear factor-kappaB (NF-κB), and activator protein (AP)-1 that are redox-sensitive factors have been highlighted [72]. We recently have demonstrated that such a compound and melatonin effectively suppress inflammation-associated colon tumorigenesis [62, 75].

Angiogenesis the formation of new blood vessels from existing vasculature has been associated with neoplasms. Angiogenesis is critical to the transition of premalignant lesions in a hyperproliferative state to the malignant phenotype, which leads to tumor growth and metastasis. The intensity of angiogenesis as assessed by counting of microvessels in neoplastic tissue acts as a prognostic factor for many solid tumors, including CRC [76, 77]. Similarly, expression of angiogenic growth factors is associated with prognosis of a variety of cancers [76, 77]. Angiogenesis enables tumors to grow larger than 1-2 mm in diameter, invade surrounding tissue, and metastasise. Angiogenesis is already targeted by chemopreventive agents at various stages of drug development or in clinical practice [76, 77]. The biomarker measured in chemoprevention must have the potential for modification by therapeutic interventional agents. In this regard, angiogenesis is a particularly attractive biomarker. There are in vivo and in vitro assays using human endothelial cell neoplasms [78], umbilical vein endothelial cells [79] and for screening potential anti-angiogenic effects of candidate chemicals. Potentially nontoxic anti-angiogenic dietary compounds include green tea polyphenols, genistein, curcumin, resveratrol, linoleic acid, hesperidin, naringenin, and allyl disulfide [80].

The defect in apoptosis mechanism is recognized as an important cause of carcinogenesis [5, 6]. A dysregulation of proliferation alone is not sufficient for cancer development as suppression of apoptotic signaling is also required. Cancer cells acquire resistance to apoptosis by overexpression of anti-apoptotic proteins and/or by the downregulation or mutation of pro-apoptotic proteins. Various studies indicate that dietary constituents, particularly phytochemicals, can modulate the complex multistage process of carcinogenesis by several mechanism(s), including apoptosis-inducing effects [81]. They include (−)-epigallocatechin gallate, curcumin, genistein, indole-3-carbinol, resveratrol, isothiocyanates, luteolin, lycopene, caffeic acid, apigenin, sylymarin, gingerol, and capsaicin [81].

3. Mechanistic Screening Assays for Detecting Potential Chemopreventive Compounds

Potential chemopreventive agents are systematically screened in a battery of short-term assays which determine the inhibition or induction of biochemical and molecular processes involved in carcinogenic processes. As summarized in Table 2, these mechanistic-based assays are roughly divided into three major categories: (i) antimutagenesis assays which evaluate carcinogenesis blocking activities, (ii) antiproliferative and antiprogression screening assays, and (iii) assays assessing antioxidant and anti-inflammatory mechanisms.

In addition to these assays, studies are in progress to establish assays utilizing DNA microarray and proteomics to

Table 2: Various assays for chemopreventive mechanisms.

Categories	Assays	Culture cells or enzymes	Measurements (effects)
Antimutagenesis	B(*a*)P-DNA adduct formation	Bronchial cells (human)	DNA damage (inhibition)
	NAD(P)H:quinone reductase	Liver cells (human)	Detoxification (induction)
	GSH *S*-transferase	Liver cells (human)	Detoxification (induction)
	GSH synthesis & GSSG reduction	Liver cells (rat)	Detoxification (induction)
Antiproliferation	TPA-induced ODC	Tracheal epithelial cells (rat)	Proliferative activity (inhibition)
	Normal epithelial cell proliferation	Primary keratinocytes (human)	Proliferative activity (inhibition)
	Poly(ADP-ribose)polymerase	Primary fibroblasts (human)	DNA damage (inhibition)
	Calmodulin regulated phosphodieaaterase	Leukemia cells (HL60)	Signal transduction regulation (inhibition)
	TPA-induced tyrosine kinase	Leukemia cells (HL60)	Signal transduction regulation (inhibition)
	EGFR	A431 (human) and 3T3 (mouse) cells	Signal transduction regulation (inhibition)
	ras farnesylation	Brain farnesyl transferase (rat)	Signal transduction regulation (inhibition)
	HMG-CoA reductase	Liver HMG-CoA reductase (rat)	Signal transduction regulation (inhibition)
	Steroid aromatase	PMSG-stimulated ovarian aromatase (rat)	Estrogenic activity (inhibition)
	Estrogen receptor	Breast cancer cells (MCF-7)	Estrogenic activity (inhibition)
	5α-reductase	Prostate 5α-reductase (rat)	Androgenic activity (inhibition)
	Cell differentiation	Leukemia cells (HL60)	Differentiation (induction)
	DNA fragmentation	Leukemia cells (HL60) or histiocytic lymphoma cells (U937 cells)	Apoptosis (induction)
Antioxidant/Anti-inflammation	AA metabolism	Macrophages/keratinocytes (human)	Anti-inflammatory activity (AA metabolism inhibition)
	TPA-induced active oxygen	Leukemia cells (HL60)	Active oxygen (inhibition)
	COX-2	Placental COX-2 (sheep)	Anti-inflammatory activity (COX-2 inhibition)
	5-LOX	RBL-1 cells (rat)	Anti-inflammatory activity (5-LOX inihibition)

AA, arachidonic acid; B(*a*)P, benzo[*a*]pyrene; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; GSH, glutathione; GSSG, oxidized glutathione; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOX, lipoxygenase; PMSG, pregnant mares' serum gonadotropin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

facilitate the discovery of new chemopreventive agents and novel molecular mechanisms of action [82, 83]. Such new emerging technologies allow the screening and monitoring of the expression levels of thousands of genes simultaneously [84]. More importantly, the technology to make customized gene chips with specific genes is also possible. In addition to monitoring the alterations in gene expression patterns in tissues undergoing carcinogenesis, these chips can be utilized to evaluate subjects at risk, such as those carrying specific germline mutations and genetic polymorphisms [85].

4. In Vitro Efficacy Model Systems

Besides the mechanistic assays mentioned above, the systematic evaluation of cancer preventive agents includes screening compounds in short-term in vitro screens which aim to select agents for subsequent whole animal testing with insight into potential mechanisms of action. Use of primary cultured cells without aneuploidy is ideal because they possess relatively intact drug metabolizing systems and normal gene numbers. Epithelial cells used are rat

tracheal epithelial cells, human lung cells, hyperplastic alveolar nodules in mouse mammary gland organ cultures, JB6 epidermal cells, and human foreskin epithelial cells. In each assay, the substances are tested over a wide range of concentrations to determine EC_{50} values. The assays include (i) a rat tracheal epithelial cell (RTE) assay which measures the ability of candidate chemopreventive agents to block the benzo[*a*] pyrene (B[*a*]P)-induced transformation of primary RTE cells [86]; (ii) an anchorage independence assay which is an effective method for detecting compounds that block carcinogenesis in the postinitiation stages and evaluates inhibition of anchorage independence in human lung tumor (A427) cells [87]; (iii) a mouse mammary gland organ cultures (MMOCs) assay which assesses the inhibitory activity of test chemicals on the development of carcinogen-induced hyperplastic alveolar nodules (HANs) in MMOC [88]. This assay is similar in appearance to the alveolar nodules produced in mouse mammary glands in vivo [89]; (iv) an in vitro assay for antipromoters or antiprogressors which is designed to identify chemopreventive agents effective in the promotion or progressive stages of carcinogenesis in JB6 epidermal cells [90]; and (v) a human foreskin epithelial assay which determines the inhibitory potential of chemopreventive agents in blocking cell growth stimulation induced by the carcinogen propane sulfone [91].

5. In Vivo Short-Term Screening Assays

This type of short-term assays identifies agents that might block or arrest carcinogenesis in the early stages. Two experimental models, which reflect major cancers in humans, are being used. They include the rat and mouse colorectal aberrant crypt foci (ACF) assay [11, 12, 92, 93] and a rat model of breast ductal carcinoma in situ model (DCIS) [94].

5.1. ACF Assay. The ACF assay is a short-term model which can identify agents that may be effective in preventing CRC [11, 12, 92, 93]. ACF, which were first described by Bird [95], are putative preneoplastic lesions consisting of aggregates of single and multiple crypt cells that exhibit hyperplasia and/or dysplasia and are thought to be the earliest detectable lesions of CRC [96–99]. Two different protocols have been developed: one which identifies compounds that inhibit initiation and a second treatment schedule which evaluates potential chemopreventive agents during the postinitiation phase of colorectal carcinogenesis. Details of these regimens have been described previously [11, 12, 100]. In the former (the initiation protocol), rats are given a test agent in the diet one week prior to the administration of a colonic carcinogen, such as AOM and continuing throughout the five-week study period. In the latter regimen (the postinitiation protocol), rats are first treated with AOM, followed four weeks later by a test agent, which is given for additional weeks. Animals are sacrificed and the ACF frequency is determined by microscopic evaluation.

5.2. DCIS Assay. The DCIS assay provides both toxicity and efficacy data for identifying candidate chemopreventive agents prior to testing in common mammary carcinogenesis models. Therefore, the induction of mammary tumorigenesis is initiated in weanling female SD rats by the intraperitoneal (*i.p.*) injection of the carcinogen *N*-methyl-*N*-nitrosourea (MNU) [94]. In general, test agents are administered in the diet, starting one week after the carcinogen administration, and thereafter are continued until the termination of the study (45–50 days later). Mammary tissue specimens are excised and processed for histopathological analysis. The efficacy is estimated as the percent reduction in the number of DCIS lesions in comparison to controls that receive a carcinogen alone.

6. Animal Efficacy Assays

The use of animal efficacy models to establish organ specificity and to generate dose-response, toxicity, and other pharmacological data is a crucial component of the determinant process for chemoprevention agents. These assays with thetoxicity tests are used for decisions regarding recommendations for clinical use. Numerous animal models are used to study inhibition of chemical carcinogenesis in rodents. Important criteria considered in selecting an in vivo model for screening cancer chemoprevention agents include (i) short study duration and induction of carcinogenesis; (ii) target-specific experimental model evidenced by the induction of cancer in the target tissues comparable in such factors as histological type and hormone dependence to those found in humans; (iii) evaluation of in vitro mechanistic activities, efficacy profiles, and relevant published data prior to the selection of models for a given possible chemopreventive agent. Typically, test agents are administered in the diet unless problems with stability are encountered. During the course of chemoprevention studies a maximum tolerated dose (MTD), defined as the highest dose level that does not cause ≥10% reduction or gain in body weight over a six-week period, is determined. The treatment schedules include the administration of test agents either before, concurrently, or following exposure to the carcinogen. Efficacy is based upon the percent inhibition of tumor incidence and/or multiplicity, or increased tumor latency in comparison to carcinogen-treated controls. Representative carcinogenesis models are listed in Table 3.

6.1. Head and Neck Carcinogenesis Models. Several well-established models of oral and respiratory tract cancer have been developed. In the hamster buccal pouch model [101], the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) is topically applied over a 12-week period, thus resulting in buccal pouch squamous cell carcinomas [102]. Rats [4] and mice [103] develop tongue cancers when exposed to 4-nitroquinoline 1-oxide (4-NQO) [104]. In rats, tongue squamous cell carcinomas and dysplasia can be induced by 4-NQO in drinking water (20 ppm) for 8 weeks [4]. Tongue dysplasia occurs during 4-NQO treatment and the incidence of tongue squamous cell carcinoma is over 50% at 32 weeks after the exposure. In this model, test chemicals can be orally administered either before, during,

Table 3: Preclinical animal models for identifying chemoprevention efficacy.

Target tissues	Species and carcinogens	Induced tumors
Oral cavity: tongue or buccal pouch	Hamster (buccal pouch): DMBA* Mouse (tongue): 4-NQO Rat (tongue): 4-NQO	SCC, PAP
Colon	Mouse: AOM, DMH, MAM acetate Rat: AOM, DMH, MAM acetate, MNU	ADC, AD, ACF
Esophagus	Rat: Nitrosaminea (MNAM, NMBA), 4-NQO Mouse: 4-NQO	SCC, PAP
Forestomach	Mouse: B(*a*)P	SCC, PAP
Liver	Mouse: various Rat: 2-AAF, DEN, DMN, 3′-Me-DAB	HCC, AD
Lung	Mouse: B(*a*)P, DMBA, NNK, Urethane, 4-NQO Hamster: DEN, MNU (trachea)	SCC, ADC, AD
Breast	Mouse: DMBA Rat: DMBA, MNU	ADC, AD, fibroadenoma
Pancrease	Hamster (duct cell)): BOP Rat (acinar cell): azaserine	ADC, AD, acinar cell carcinoma
Skin	Mouse: UV radiation, B(*a*)P/TPA, DMBA, DMBA/TPA, MC	SCC, PAP
Glandular stomach	Rat: MNNG, MNU	ADC
Urinary bladder	Mouse: OH-BBN Rat: MNU, OH-BBN	TCC

2-AAF, 2-acetylaminofluorene; ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; B(*a*)P, benzo[*a*]pyrene; BOP, *N*-bis(2-oxopropyl)nitrosamine; DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMH, 1,2-dimethylhydrazine; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; MAM acetate, methylazoxymethanol acetate; MC, 3-methylcholanthrenel; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; MNAN, *N*-methyl-*N*-amylnitrosamine; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NMBA, *N*-nitrosomethylbenzylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-NQO, 4-nitroquinoline 1-oxide; OH-BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; PAP, squamous cell papilloma; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

or following 4-NQO exposure [4]. For the induction of lung adenosquamous cell carcinoma or squamous cell carcinoma of hamsters, MNU is administered over a 15-week period, thus resulting in approximately 40–50% of the treated animals after 6 months [105]. Starting one week prior to MNU exposure, a test compound is administered over 180 days. In the second model, hamsters are given the carcinogen diethylnitrosamine (DEN), subcutaneously, twice per week over a 20-week period, resulting in the formation of lung adenocarcinomas in about 50% and tracheal tumors in over 90%, of treated animals [106]. As in the MNU model, test agents are given prior to carcinogen exposure. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) can induce lung tumors in A/J mice and a large number of compounds are now available that inhibit the mouse lung tumorigenesis induced by NNK [107]. Another lung cancer bioassay is the strain A mouse model [108]. Strain A mice spontaneously develop lung tumors as early as 3-4 weeks, with lung tumor incidences approaching 100% at 24 months of age. A/J mice are also utilized for studies on colon tumorigenesis because of their susceptibility to colonic carcinogens [109].

6.2. Colorectal Carcinogenesis Models. Potential inhibitors of colorectal carcinogenesis can be assessed utilizing models in both rats and mice [11, 12, 110, 111]. According to established protocols, 1,2-dimethylhydrazine (DMH), AOM, or methylazoxymethanol (MAM) acetate is administered intraperitoneally or subcutaneously, thus resulting in colorectal adenocarcinoma development within 32–40 weeks in either species. DMH is first activated to form AOM and then is metabolized by the liver to form MAM, the ultimate carcinogen, which is excreted via glucuronide conjugation. In the AOM induction model, a single or multiple (up to 3 times) subcutaneous dose of AOM in male F344 rats results in the occurrence of colorectal adenocarcinoma and adenoma in approximately 70% of treated animals by 40 weeks. Again, the test agents can be orally administered either before, during, or following carcinogen treatment [112]. In comparison to rats, mice should receive multiple exposures of a colonic carcinogen to induce colonic tumors and tumor development needs long-term period. A mouse model recently established for colorectal carcinogenesis [113], in which different colonic carcinogens are followed by a colitis-inducing agent, dextran sodium sulfate (DSS),

is quite useful to identify potential chemopreventive agents within a short-term period [68, 114].

6.3. Mammary Carcinogenesis Models. Chemopreventive efficacy against mammary gland carcinogenesis is routinely assessed by either the MNU- or DMBA-induced models [115]. Both protocols utilize female SD rats and require that the carcinogen is given as a single dose at 50 days of age. In some instances, the carcinogen is administered to older animals (180 days) which is more representative of the human target population. Tumor incidences at 120 days after carcinogen treatment are similar, ranging from 80–100% in the DMBA protocol and 75–95% in the MNU model. However, the histological types of tumors induced by the two carcinogens are different. DMBA-induced mammary tumors are predominantly adenoma and fibroadenoma, with some adenocarcinoma, whereas MNU-induced mammary tumors are invasive adenocarcinomas. The chemopreventive activity of the test agents is determined by the percent reduction in tumor incidence or percent increase in tumor latency relative to controls treated with the carcinogen alone. These models produce hormonally responsive tumors. In addition to these chemically-induced mammary carcinogenesis models, several genetically engineered animals have been introduced to investigate breast carcinogenesis and evaluate the efficacy of candidate chemopreventive agents. They include a COX-2 overexpressing mouse model [116], a *Ras*-driven mouse mammary tumorigenesis model [117], and a HER-2/neu transgenic mouse model [118]. In addition, mammary stem cell models can be used for studying how the hormonally regulated paracrine interactions influence stem cells and the stem cell niche during mammary carcinogenesis [119]. Another interesting model system for understanding normal human breast development or tumorigenesis is the orthotopic xenograft model that has the potential to improve the understanding of crosstalk between tissue stroma and the epithelium as well as factors involved in breast stem cell biology tumor initiation and progression [120].

6.4. Urinary Bladder Carcinogenesis Models. Urinary bladder neoplasms are typically induced by the carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN) which can induce invasive transitional cell carcinomas morphologically similar to those found in humans [121, 122]. This carcinogen is given either intragastrically or in drinking water over an 8-week period to 50-day old BDF mice (C57BL/6 × DBS/2-F_1) or F344 rats, thus resulting in a 40–50% incidence of bladder tumor incidence at 180 days after OH-BBN treatment. Treatment schedules for a test agent administration are as described above [123].

6.5. Skin Carcinogenesis Models. Agents effective in inhibiting skin carcinogenesis are identified in a two-stage skin carcinogenesis model utilizing DMBA and TPA, which are applied topically to the back skin of SENCAR or CD-1 mice [124, 125]. Both strains of mice are highly susceptible to skin tumor induction. Skin papillomas appear as early as 6 weeks postcarcinogen treatment, eventually progressing to squamous cell carcinomas by 18 weeks [126]. Test agents are generally administered in the diet or in some experiments are topically applied according to several predefined treatment regimens. As to melanoma chmoprevention study, we should read an elegant review for new perspectives of this research area [127]. In addition to in vitro screening assay [128], several genetically altered animal (mouse) models of melanoma [129–132], including hepatocyte growth factor/scatter factor (HGF/SF) transgenic mice [133–136], have been introduced for prevention [137] and biology [138] of this neoplasm. Also, a three-dimensional skin reconstruction model [139] is useful for determining the therapeutic efficacy of selected chemicals or drugs in cultured melanoma cells [140]. While epidemiological studies suggest sunlight as an etiologic agent for the pathogenesis of melanoma, recent experimental investigations by the group Meyskens, Jr. indicated that elevation of reactive oxygen species follows from melanin serving as a redox generator [127] and this may be involved in the etiology and pathogenesis of cutaneous melanoma. Such findings will help to establish novel preventive and therapeutic approaches to this malignancy.

7. Transgenic and Gene-Knockout Animal Models

Animal models that mimic the specific characteristics of human carcinogenesis may prove to be a valuable resource in both evaluating chemopreventive efficacy and identifying appropriate biomarkers for measuring the chemopreventive activity. Transgenic and gene knockout mice that carry well-characterized genetic lesions predisposing them to carcinogenesis are appropriate models for chemoprevention testing (Table 4). Some of the best developed models include the multiple intestinal neoplasia (*Min*) mouse [141] and other strains possessing lesions in the *Apc* gene [142]. The *Min* mouse carries an *Apc* mutation similar to that found in human familial adenomatous polyposis (FAP) patients. These mice are predisposed to develop predominantly small intestinal adenomas, but a few in the large intestine. By manipulating two or more carcinogenesis-associated genes, such as modifier genes, in a single animal, closer approximations of human carcinogenesis may be possible. Numerous colonic tumors develop in the large bowel in *Min* mice at 3 weeks after one-week-exposure of DSS [143], thus suggesting the importance of gene-environmental interaction in cancer development [68]. It might be feasible to knock out *p53* in an animal that already carries another tumor suppressor defect such as *Apc* or p16. Recently, new transgenic animal models for mammary [144], tongue [145, 146], pancreas [147], and gall bladder [148] cancers have been reported. These models might be useful for discovering possible novel cancer chemopreventive agents.

8. Combination Treatment

One strategy for improving the efficacy and lessening toxicity is using combinations of agents [149, 150]. Synergistic or additive effects may be observed when two agents with

Table 4: Transgenic/gene KO mice or rats for identifying chemoprevention efficacy.

Genes	Target tissues	Genetic lesions	Induced tumors
Min	Intestine	Heterozygous *Apc* 2549	AD
Apc	Intestine	Heterozygous *Apc* 1638	AD
MLH1/*Apc* 1638	Intestine	Heterozygous MLH1 and *Apc* 1638	AD
Msh2/*Min*	Intestine	Heterozygous MLH2 and *Apc* 2549	AD
pim	Lymphatic system	Amplified *pim*-1	T-cell lymphoma
TG:AC	Skin	Ha-*ras* mutation	PAP, possibly carcinoma
TSG-p53	Skin	Heterozygous p53 deficient	PAP, possibly carcinoma
A/JxTSG-p53	Lung	Heterozygous p53 deficient	AD
A/JxUL53	Lung	Heterozygous p53 mutant	AD
TGFβ1	Liver, lung	Heterozygous TGFβ1 mutant	AD, carcinoma
v-Ha-*ras*	Skin	Ha-*ras* + Human keratin K-1	HP, PAP
K14-HPV16	Skin	HPV-infected (K14-HPV16 heterozygote), estradiol-treated + SV40 T-antigen	PAP, condyloma
	Cervix	HPV-infected (K14-HPV16 heterozygote), estradiol-treated + SV40 T-antigen	Dysplasia
rPB-SV40 Tag transgene	Prostate	SV40 large tumor T antigen (Tag)	Dysplasia, AD, ADC (TRAMP model)
C3(1)-SV40	Prostate	Heterozygous rat prostate steroid binding gene [C3(1)] + SV40 T-antigen	Dysplasia, AD, ADC
	Breast	Heterozygous rat prostate steroid binding gene [C3(1)] + SV40 T-antigen	ADC
ErbB-2	Gall bladder	*BK.ErbB-2*	AD, ADC
Human c-Ha-*ras*	Breast	Jcl/SD-TgN(H *ras* Gen)128Ncc	ADC
	Urinary bladder	Jcl/SD-TgN(H *ras* Gen)128Ncc	TCC
	Prostate	Jcl/SD-TgN(H *ras* Gen)128Ncc	ADC, PIN
	Tongue	Human prototype c-Ha-*ras* gene with its own promoter region	PAP, SCC

AD, adenoma; ADC, adenocarcinoma; HP, hyperplasia; HPV, human papilloma virus; PAP, squamous cell papilloma; PIN, prostate intraepithelial neoplasia; TCC, transitional cell carcinoma; TGF, transforming growth factor.

different mechanisms of action are combined [149]. Such improved activity of inhibition may allow either or both of the agents to be given at lower doses, thereby reducing the toxic potential. Examples of chemical combinations producing positive results in experimental animal models include all-*trans*-*N*-(4-hydroxyphenyl)retinamide (4-HPR) and tamoxifen in the rat mammary gland [151] and 2-dinuoromethylorinithine (DFMO) and piroxicam in the rat colon [152, 153]. The identification and evaluation of other effective agent combinations is an interesting and important research effort for chemopreventive agent development [154, 155]. Since different treatment schedules of the two drugs can be compared and evaluated for synergism, additivity, or antagonism using a quantitative method based on the median-effect principle of Chou and Talalay [156–159] can identify the ideal combination treatment for obtaining an improved effect in comparison to a single exposure of the potential chemopreventive agent [160–162].

9. Intermediate Biomarkers

Carcinogenesis proceeds through a very long preclinical period. The collective hope is that multiple opportunities exist for chemoprevention to arrest or reverse progression towards malignancy. Intermediate biomarkers of malignant neoplasms are the phenotypic, genotypic, and molecular changes that occur during carcinogenesis [69]. An important component of chemopreventive agent development research is the identification and characterization of intermediate biomarkers that may serve as surrogate end points for cancer incidence reduction in chemoprevention clinical trials [163]. Such efforts are critical to the progress of chemoprevention and have the potential for cost-effective development of chemopreventive research [5, 6, 69]. Biomarkers include proliferation, apoptosis, growth factors and their receptors, genetic alterations, and so forth in the target tissues [162, 164, 165]. In the hope of faster progress with fewer subjects and lower total cost, much effort is being expended on the search for reliable biomarkers to predict the likelihood of developing cancer and/or to signal the effectiveness of chemopreventive therapy [166]. Considerable attention has been paid to identifying those markers that can act as surrogate markers for cancer development since favorable modulation of the surrogate end-point biomarker may demonstrate the effectiveness of a putative preventive treatment. However, the complexity of the biology challenges the ability to measure the effectiveness of attempts to arrest or reverse carcinogenesis, other than through costly and

time-consuming prospective trials with the disease state as the endpoint. Despite much work, to date no prehistological biological or molecular intermediate marker has yet been validated for sporadic cancers.

For chemoprevention of prostate cancer [167] and other types of cancer, natural and synthetic agents that may suppress, reverse, or regress precancer and delay progression to invasive cancer can be used [77]. Epidemiological evidence suggests that environmental factors, such as diet, play a role in the development and progression of prostate cancer. The number of potential protective dietary compounds or whole dietary products that are indicated to have preventive effects is piling up and demands further evaluation. To face this scientific field, a strategy that combines prostate-specific antigen (PSA)-based clinical trials with experimental human xenograft studies to evaluate potential chemopreventive agents against prostatic cancer is proposed [168, 169]. PSA for prostate adenocarcinoma, even though has relatively poor specificity, is cheap and easily followed with minimally invasive procedures.

10. Future Direction

Epidemiologic evidence suggests that green tea polyphenols reduce the risk of some forms of cancer, while data for other commonly used phytochemicals is less convincing. Part of the problem lies in precise quantitation of foodborn phytochemicals where multiple dietary sources of a particular phytochemical are involved, as in recent studies of dietary quercetin on the prevention of heart disease. Future investigations may focus on specific foodborn phytochemicals and/or botanicals capable of intervening in critical pathways in carcinogenesis as indicated by the possible use of "natural" NSAIDs in the prevention of CRC and the soy-based phytoestrogens in the prevention of breast cancer. Much is to be learned by collaboration between cancer researchers and ethnopharmacologists for probing dietary prevention of cancer. In addition, the development and validation of new in vitro and in vivo assays of high predictive value for discovering agents with human chemopreventive potential is needed. Care, however, is needed when extrapolating in vitro data to in vivo models because it cannot be assumed that the effects seen when cells are exposed directly to active compounds that would be candidate chemopreventive agents will be seen when they are consumed in the diet. We should investigate whether they are capable of distribution throughout the body when they are absorbed after ingestion. The understanding of the molecular and cellular processes, such as gene and protein expression, apoptosis, angiogenesis, signal transduction, involved in carcinogenesis is rapidly increasing. Currently several translational studies using phytochemicals and bioactive compounds, such as indole-3-carbinol [170], ellagitannins [171], sulforaphane [172], lycopene [173], diallyl trisulfide [174], omega-3 fatty acids [175], proanthocyanidins [176], green tea polyphenols [177], genistein [178], silymarin [179], and curcumin [180, 181], from plants are underway.

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