2.7. Statistics

All *P*-values were generated using two-tailed Student's *t*-tests.

3. Results

3.1. Interaction of MAPO1 with FLCN and AMPK

To confirm that MAPO1 protein interacts with FLCN and AMPK, a co-immunoprecipitation experiment was performed. Whole cell extracts were prepared from mouse YT102 (*Mgmt*$^{-/-}$) cells expressing Flag-tagged MAPO1, and were subjected to immunoprecipitation using an anti-Flag antibody conjugated to agarose beads. The results are shown in Fig. 1A. With whole cell extracts, almost the same intensity of bands for FLCN and AMPKα were detected in both control and Flag-MAPO1-transfected cells. When the materials were immunoprecipitated with the anti-Flag antibody, co-precipitated FLCN and AMPKα were clearly detected, concomitant with the effective precipitation of Flag-MAPO1, whereas no such bands were seen in a sample precipitated from cells treated with the control vector alone.

To evaluate the interaction of FLCN with MAPO1 and AMPK in a reciprocal manner, whole cell extracts prepared from YT102 cells expressing FLAG-tagged MAPO1, with or without HA-tagged FLCN, were applied for immunoprecipitation using an anti-HA antibody (Fig. 1B). When the HA-tagged FLCN was precipitated, as indicated by doublet bands by the immunoblotting analysis, the Flag-tagged MAPO1 and AMPKα were co-precipitated. It is evident, therefore, that MAPO1 interacts with FLCN and AMPK in mouse cells.

3.2. Suppression of the induction of apoptosis in Flcn- and Ampkα-knockdown cells

Since MAPO1 has been identified as an apoptosis-inducing protein, it is plausible that the MAPO1-bound proteins, FLCN and AMPK, might also be involved in apoptosis induction. To examine the possible roles of these proteins, siRNAs specific for the *Flcn* or *Ampkα* genes were introduced into YT102 (*Mgmt*$^{-/-}$) cells. As shown in Fig. 2A and B, two independent siRNAs (siFlcn#1 and #2, and siAmpkα#1 and #2), designed at different sequences of each gene, effectively suppressed the expression of the genes when measured at 48 h after their introduction. The expression level of the *Mapo1* gene in siMapo1-treated cells also decreased to 43% of that in cells that were treated with the control RNA, siCont, as measured by quantitative real time PCR [16]. To monitor the appearance of cells with sub-G_1 DNA content, cells were treated with or without 0.4 mM MNU for 1 h and subjected to a flow cytometric analysis 72 h later. After treatment with MNU, the sub-G_1 cell population increased to more than 20% in the siCont-treated cells (Fig. 2C). Under the same conditions, the degrees of the increases in the cells treated with siRNAs against the *Flcn*, *Ampkα* and *Mapo1* genes were significantly suppressed. These results favor the notion that FLCN and AMPKα, as well as MAPO1, are involved in MNU-induced apoptosis through protein interactions.

3.3. Suppression of the induction of apoptosis by an AMPK inhibitor

The effects of *Ampkα* knockdown on the MNU-induced apoptosis were further examined at multiple time points. The YT102 cells transfected with siCont or siAmpkα#2 were exposed to 0.4 mM MNU for 1 h and then subjected to a flow cytmetric analysis. As shown in Fig. 3A, the sub-G_1 cell population increased gradually, with similar kinetics in cells transfected with either type of siRNA, but the degree of the increase in cells transfected with siAmpkα was significantly lower than that of siCont-transfected cells.

To obtain further evidence supporting the involvement of AMPK in MNU-induced apoptosis, compound C, a specific inhibitor of AMPK, was used to downregulate the function of AMPK. YT102 cells were exposed to 0.4 mM MNU for 1 h, followed by incubation with or without 2 μM of compound C for 72 h, and then cells were subjected to a flow cytometric analysis. As shown in Fig. 3B, the sub-G_1 cell population in compound C-treated cells after MNU treatment significantly decreased in comparison to those not treated with the inhibitor. The inhibitory effects of compound C on AMPK activity were assessed by immunoblotting using an antibody that specifically recognizes a phosphorylated form of AMPKα, since AMPK is activated when the catalytic subunit of AMPKα becomes phosphorylated [27–29]. As shown in Fig. 3C, AMPK appeared to be activated after MNU treatment, while such activation was significantly suppressed by the exposure of cells to compound C. These findings are consistent with the notion that AMPK plays an important role in the induction of apoptosis triggered by MNU.

3.4. MAPO1- and FLCN-dependent activation of AMPK during the induction of apoptosis

To further examine if AMPKα is phosphorylated during the induction of apoptosis, YT102 cells were treated with 1 mM MNU and then collected at 0, 24, 48 and 72 h after treatment. Under these conditions, apoptosis was effectively induced, as was evident by the detection of the mitochondrial membrane depolarization and the caspase-3 activity [16]. The whole cell extracts were prepared, and the phosphorylation levels of AMPKα were assessed by

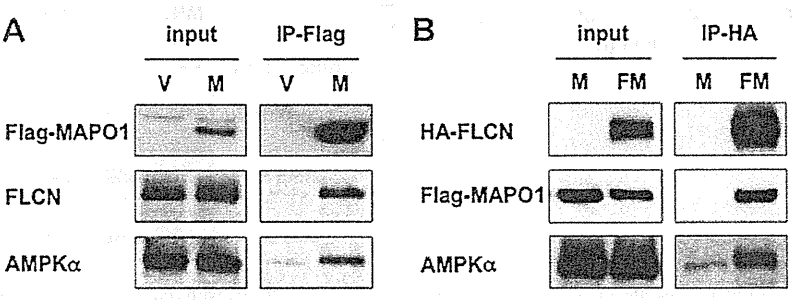

Fig. 1. The association of MAPO1, FLCN and AMPKα proteins. (A) The interaction of MAPO1 with FLCN and AMPKα. YT102 cells were transfected with the pIRES-puro3 vector (termed as V) or pIRES-puro3 containing Flag-tagged *Mapo1* cDNA (termed as M) and harvested after incubation for 24 h. Whole cell extracts (input) were used for immunoprecipitation using anti-Flag M2 antibody beads (IP-Flag). The materials were subjected to SDS-PAGE, transferred to a membrane and immunoblotted using antibodies that recognize the Flag-tag, FLCN and AMPKα. (B) The interaction of FLCN with MAPO1 and AMPKα. YT102 cells were transfected with either pIRES-puro3 containing Flag-tagged *Mapo1* cDNA (termed as M) or pIRE-puro2 carrying HA-tagged *Flcn* cDNA and pIRES-puro3 containing Flag-tagged *Mapo1* cDNA (termed as FM) and were harvested 24 h later. Following immunoprecipitation using anti-HA HA7 antibody beads (IP-HA), an immunoblotting analysis was performed as described in (A) with anti-HA, anti-Flag and anti-AMPKα antibodies.

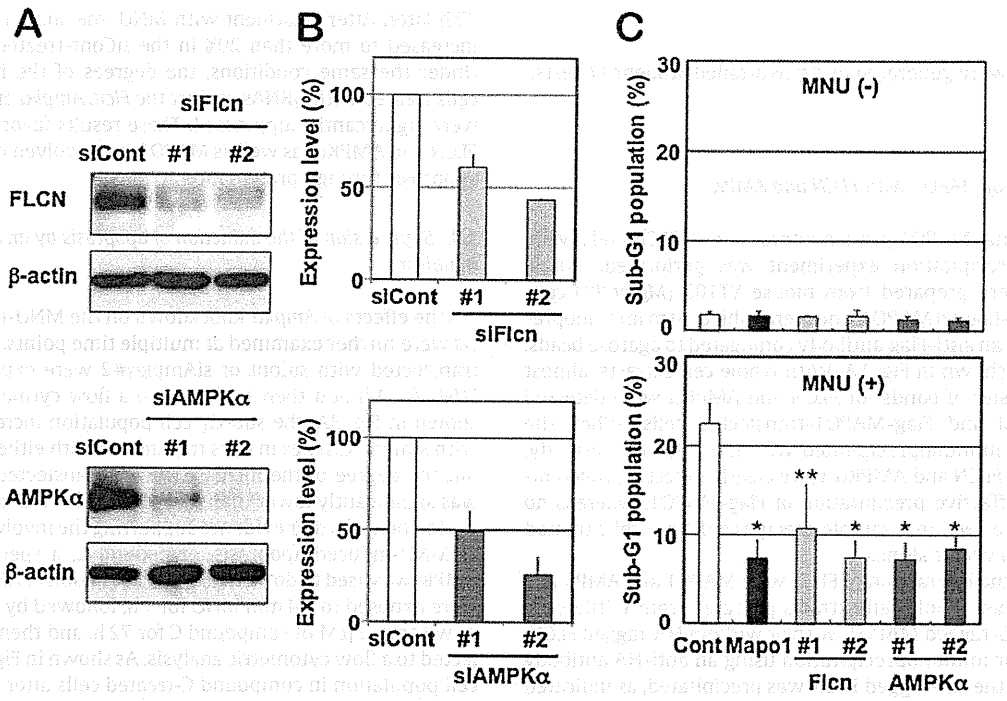

Fig. 2. The suppression of apoptosis by siRNAs targeting the three types of genes. (A) The expression levels of FLCN and AMPKα in cells treated with siRNAs. The whole extracts of YT102 cells transfected with control and two independent siRNAs specific for the corresponding genes were used for the immunoblotting analysis with antibodies specific for FLCN, AMPKα and β-actin (loading control). (B) The relative expression levels of FLCN and AMPKα in the cells treated with siRNAs, as measured by an immunoblotting analysis in (A). (C) The sub-G_1 population of cells transfected with control, *Mapo1*-, *Flcn*- or *Ampkα*-siRNA after MNU treatment. Two days after transfection with siRNA, YT102 cells were treated with or without 0.4 mM MNU for 1 h and then incubated for three days. The cells were harvested and subjected to a flow cytometric analysis. $^*P < 0.01$; $^{**}P < 0.05$ when comparing the sub-G_1 populations in the control and gene-specific siRNA-transfected cells.

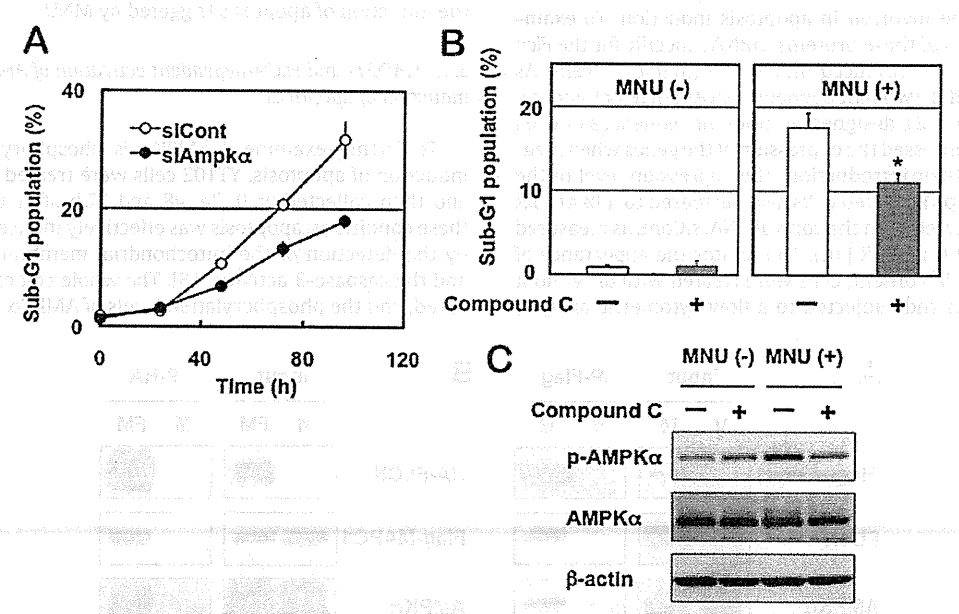

Fig. 3. The involvement of AMPK in MNU-induced apoptosis. (A) The sub-G_1 population of cells transfected with control or *Ampkα* siRNA after MNU treatment. Two days after transfection with siRNA, the YT102 cells were treated with 0.4 mM MNU for 1 h and then harvested at 0, 24, 48, 72 and 96 h after MNU treatment, and subjected to a flow cytometric analysis. The numbers of the cells in the sub-G_1 population were counted and the ratios were plotted. Open circles, siCont-transfected cells; closed circles, siAmpkα-transfected cells. (B) The suppression of apoptosis by an AMPK inhibitor. After treatment with or without 0.4 mM MNU for 1 h, YT102 cells were incubated in medium supplemented with or without 2 μM compound C for three days. The cells were then harvested and subjected to a flow cytometric analysis to monitor the sub-G_1 population of cells. $^*P < 0.01$ when comparing the sub-G_1 populations in compound C-untreated and compound C-treated cells after exposure to MNU. (C) The inhibition of the AMPK activity by compound C. The whole cell extracts from the cells harvested at 48 h after MNU treatment were subjected to an immunoblotting analysis using antibodies that recognize phospho-AMPKα (Thr172), AMPKα and β-actin, respectively.

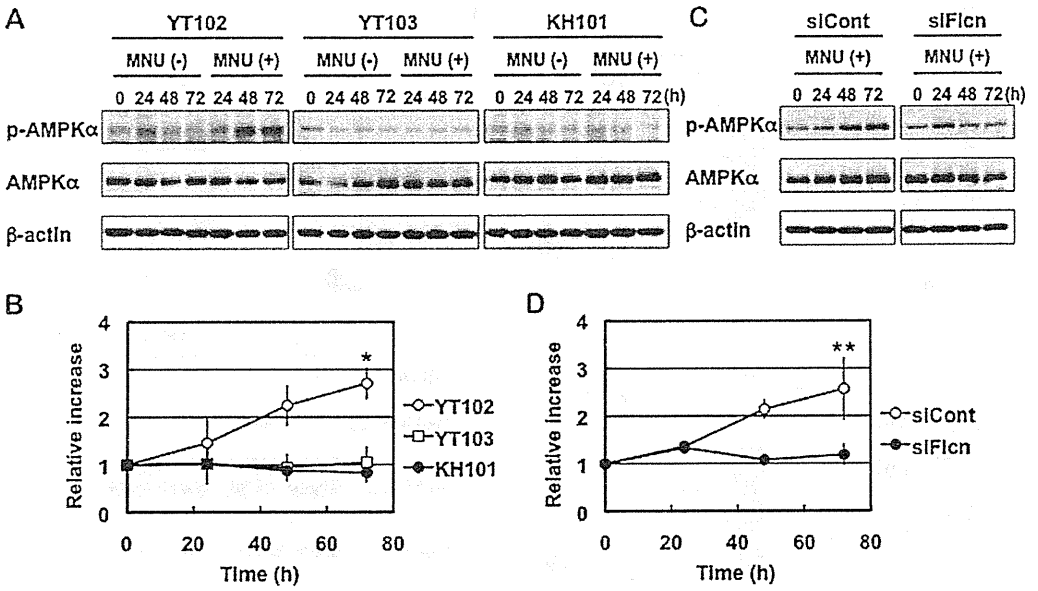

Fig. 4. The activation of AMPK after MNU treatment. (A) The phosphorylation of AMPKα in cells with different genetic backgrounds. Three cell lines, YT102 (*Mgmt*$^{-/-}$), YT103 (*Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$) and KH101 (*Mgmt*$^{-/-}$ *Mapo1*$^{+/-}$), were treated with or without 1 mM MNU for 1 h and then incubated for 0, 24, 48 or 72 h. The whole cell extracts from cells harvested at various times after MNU treatment were subjected to an immunoblotting analysis using antibodies that recognize phospho-AMPKα (Thr172), AMPKα and β-actin, respectively. (B) The relative intensities of the bands for phospho-AMPKα (Thr172) after MNU treatment. Open circles, YT102; open squares, YT103; closed circles, KH101. *$P < 0.01$ when comparing the relative intensities for YT102 cells with those of the YT103 and KH101 cells at 72 h after exposure to MNU. (C) Activation of AMPK in cells transfected with *Flcn*-siRNA. Two days after transfection with control or *Flcn*-siRNA, the YT102 cells were treated with or without 1 mM MNU for 1 h. The analysis was performed as described above. (D) The relative intensities of bands for phospho-AMPKα (Thr172) after MNU treatment. Open circles, siCont-transfected cells; closed circles, siFlcn-transfected cells. **$P < 0.05$ when comparing the relative intensities of the control and *Flcn*-specific siRNA-transfected cells at 72 h after exposure to MNU.

an immunoblotting analysis. As shown in Fig. 4A and B, the levels of phosphorylation of AMPKα increased gradually and reached about 2.7-folds at 72 h after MNU treatment, whereas no such increase was observed in cells not expose to MNU. The amounts of the AMPKα protein were almost constant under these situations. In YT103 (*Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$) cells, which are unable to induce apoptosis due to their lack of the *Mlh1* gene, the increase of phosphorylated forms of AMPKα was hardly detectable, even after MNU treatment. These results indicate that AMPK is activated during the course of the induction of apoptosis, triggered in a mismatch repair protein-dependent manner. To evaluate the effects of *Mapo1* mutation on the activation of AMPK, we used KH101 (*Mgmt*$^{-/-}$ *Mapo1*$^{+/-}$) cells, which carry an insertional mutation in one of the alleles of the *Mapo1* gene and exhibit haploinsufficiency for the induction of apoptosis triggered by MNU treatment [16]. Similar to the results described above, no increase in the band corresponding to phosphorylated AMPKα was detected even after treatment with MNU (Fig. 4A and B). Since MAPO1 interacts with FLCN (Fig. 1), it was supposed that FLCN might also play a role in the activation of AMPK during the course of apoptosis. To examine this possibility, YT102 (*Mgmt*$^{-/-}$) cells were transfected with siRNA targeting the *Flcn* gene (siFlcn#2), and then were exposed to 1 mM MNU for 1 h. The immunoblotting analyses of these samples collected after incubation for 0, 24, 48 and 72 h revealed that phosphorylation of AMPKα, which occurred gradually in siCont-transfected cells, did not take place in the siFlcn-transfected ones (Fig. 4C and D). These results indicate that the activation of AMPK, which occurs during the course of MNU-induced apoptosis, is dependent on the functions of both FLCN and MAPO1.

3.5. Induction of apoptosis through activation of AMPK

To confirm the importance of the activation of AMPK for the induction of apoptosis, AICA-Ribose (AICAR), a specific activator of AMPK, was applied to YT102 cells. After treatment with a low dose (0.2 mM) of AICAR for 48 h, the viabilities of cells were analyzed, based on the trypan blue exclusion assay. As shown in Fig. 5A, there was a significant increase of trypan blue staining-positive cells after treatment with AICAR in the YT102 (*Mgmt*$^{-/-}$ *Mapo1*$^{+/+}$) cells, whereas no such increase was observed in the *Mapo1*-defective KH101 (*Mgmt*$^{-/-}$ *Mapo1*$^{+/-}$) cells even after the same treatment. To determine if the increase in dead cells was related to the induction of apoptosis, the cells were subjected to an assay for mitochondrial membrane depolarization, which is known to occur during the process of apoptosis. The results are shown in Fig. 5B and C. The depolarization of the mitochondrial membrane was induced after treatment with AICAR in YT102 cells, but not in *Mapo1*-defective KH101 cells. The results indicate that the function of MAPO1 is necessary for AICAR-induced apoptosis. An immunoblotting experiment, the results of which are shown in Fig. 5D, revealed that the AICAR-treatment induced phosphorylation of AMPKα to the similar level to that when treated with MNU, however, such an induction did not occur in the *Mapo1*-defective KH101 cells. These results suggest that the activation of AMPK is important for the induction of apoptosis, and that a normal level of MAPO1 is necessary for the activation of AMPK.

We next examined if FLCN, which interacts with MAPO1, is also required for the AICAR-induced cell death. For this study, we applied AICAR to YT102 cells whose FLCN function was knocked down by siRNA (siFlcn#2). As shown in Fig. 6A–C, the degree of AICAR-induced cell death, which was accompanied by the depolarization of the mitochondrial membrane, was significantly lower in siFlcn-transfected cells as compared to that in siCont-transfected ones. Furthermore, the AICAR-induced AMPKα phosphorylation was almost completely blocked in siFlcn-transfected cells (Fig. 6D). Therefore, these results suggest that FLCN is required for AMPK activation, as well as the cell death induced by the treatment with AICAR.

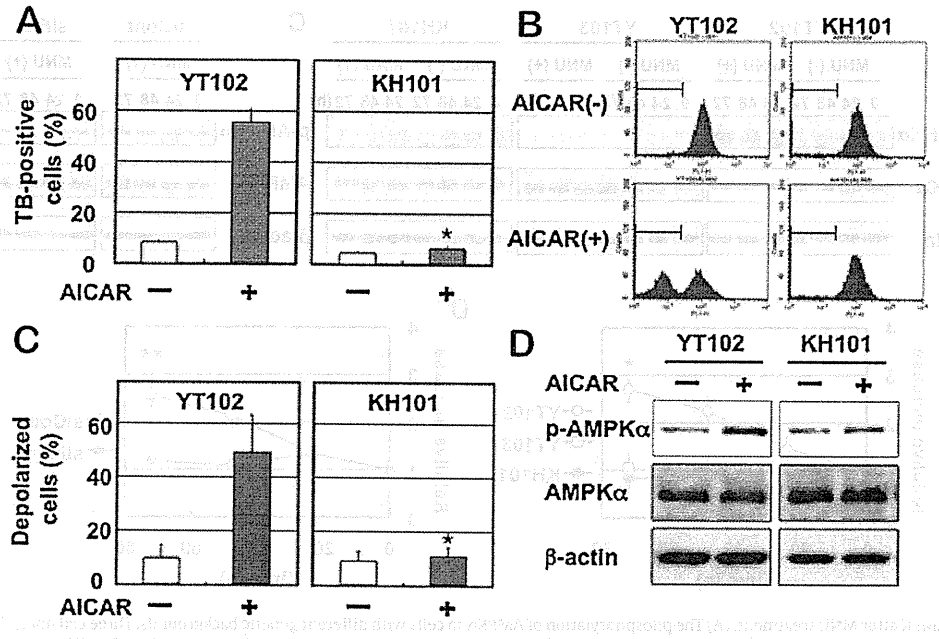

Fig. 5. MAPO1-dependent cell death induced by an AMPK activator. *Mapo1*-proficient YT102 and *Mapo1*-defective KH101 cells were incubated in a medium supplemented with or without 0.2 mM AICAR for two days and then harvested. (A) The viabilities of the cells. The numbers of cells stained with trypan blue (TB) were counted and the ratios are shown. $^*P < 0.01$ when comparing the TB-positive YT102 and KH101 cells after exposure to AICAR. (B) Depolarization of the mitochondrial membrane. The cells were evaluated by a mitochondrial membrane depolarization assay, and representative patterns of the assay are shown. The populations of depolarized cells were gated by bars. (C) The levels of mitochondrial membrane depolarization. The mean values obtained from three independent experiments in (B) and the standard deviations (bars) are presented. $^*P < 0.01$ when comparing the depolarized cells in YT102 and KH101 cells after exposure to AICAR. (D) Activation of AMPK after treatment with AICAR. The whole cell extracts prepared from cells, treated with or without AICAR, were subjected to an immunoblotting analysis using antibodies specific for phospho-AMPKα (Thr172), AMPKα and β-actin, respectively.

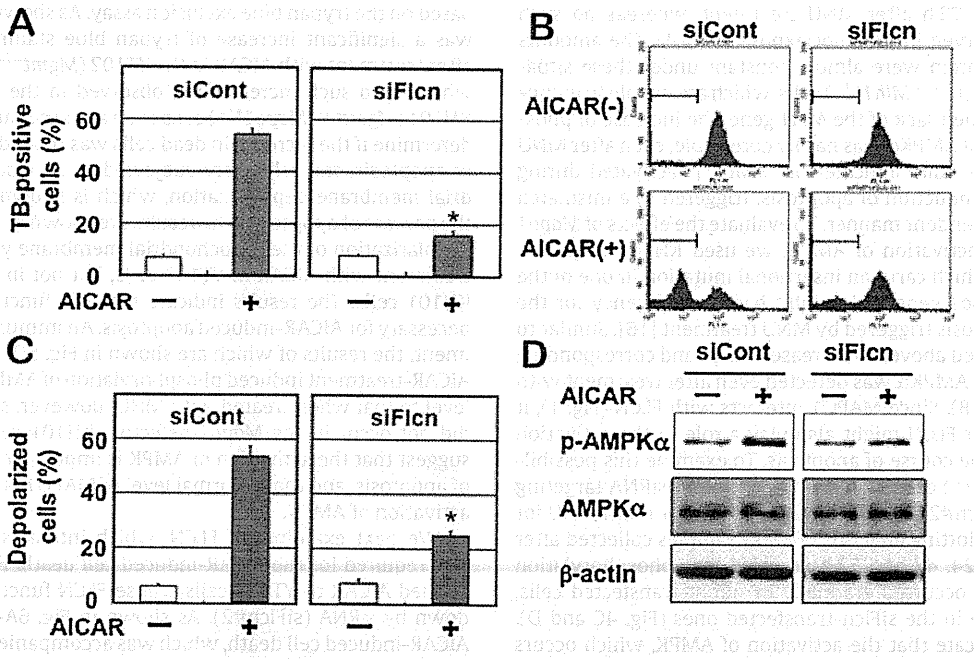

Fig. 6. FLCN-dependent cell death induced by an AMPK activator. YT102 cells transfected with control- or *Flcn*-siRNA were cultured with or without 0.2 mM AICAR for two days and then harvested. (A) The viabilities of the cells. The numbers of cells stained with trypan blue (TB) were counted and the ratios are shown. $^*P < 0.01$ when comparing the TB-positive siCont-transfected and siFlcn-transfected cells after exposure to AICAR. (B) Depolarization of the mitochondrial membrane. The cells were evaluated by a mitochondrial membrane depolarization assay, and representative patterns of the assay are shown. The populations of depolarized cells were gated by bars. (C) The levels of mitochondrial membrane depolarization. The mean values obtained from three independent experiments in (B) and the standard deviations (bars) are presented. $^*P < 0.01$ when comparing the depolarized cells in siCont-transfected and siFlcn-transfected cells after exposure to AICAR. (D) Activation of AMPK after treatment with AICAR. The whole cell extracts prepared from AICAR-treated or -untreated cells, were subjected to an immunoblotting analysis using antibodies specific for phospho-AMPKα (Thr172), AMPKα and β-actin, respectively.

4. Discussion

MAPO1 was identified as one of the protein elements functioning at a certain step following the induction of apoptosis [16]. In *Mapo1*-defective cells, mitochondrial membrane depolarization and caspase-3 activation were not observed even after exposure to MNU, although the cells retain the ability for mismatch repair protein-dependent DNA damage detection and signaling. Subsequent studies have revealed that MAPO1 is identical to FNIP2 and FNIPL, reported by Hasumi et al. [23] and Takagi et al. [24], respectively. This protein is bound to folliculin, encoded by the *FLCN* tumor suppressor gene, and AMP-activated protein kinase (AMPK). To analyze the possible roles of folliculin and AMPK in the induction of apoptosis, we introduced siRNAs specific for the *Flcn* or *Ampkα* gene and then treated the cells with MNU. The flow cytometric analyses performed to measure the sub-G_1 population of cells revealed that folliculin and AMPK, as well as MAPO1, were involved in MNU-induced apoptosis. Taken together, these data suggest that MAPO1 forms a protein complex(es) with folliculin and AMPK, and plays a role in a signal transduction pathway of apoptosis.

It is known that AMPK is one of the signaling kinases that negatively regulates cell growth and proliferation and is phosphorylated itself under conditions of energetic stress [26–29]. Several recent papers have observed the pro-apoptotic potential of activated AMPK [30–33]. In this report, we found a gradual increase in the levels of AMPK phosphorylation in *Mapo1*-proficient cells after MNU treatment, implying a possible involvement of the activation of AMPK in the MNU-induced apoptosis pathway. In *Mapo1*-deficient cells, AMPK activation in this manner was hardly detectable, even after the treatment with MNU. Furthermore, the treatment of cells with AICAR, a specific activator of AMPK, resulted in AMPKα phosphorylation and mitochondrial membrane depolarization in a *Mapo1*-dependent manner. These findings extended onto the case of *Flcn*-knockdown cells. Taken together, it is likely that MAPO1 and FLCN positively regulate the activation of AMPK through their mutual interaction in the apoptotic signaling pathway, triggered by an alkylating agent. MAPO1 and FLCN proteins have been reported to undergo some modifications in cells [17,24]. The treatment with an alkylating agent might affect the modified states of these proteins, and might cause the activation of the protein complex, thus leading to AMPK activation. Another folliculin-interacting protein, FNIP1, which is homologous to MAPO1, is also capable of binding to AMPK [17]. The activation of AMPK might therefore be regulated in more complex ways under the balance of MAPO1 and FNIP1 activities.

Another important problem which remains to be solved is how the AMPK–MAPO1–FLCN complex is activated by the signal delivered from the mismatch repair protein complex, which itself is activated through the interaction with DNA carrying base mismatches. The signal may be delivered by direct physical contact between the two complexes or through the involvement of other protein factors. The protein linking analyses, aided by mass spectrometry, have been performed, but no evidence to show the physical association of the two complexes was obtained (unpublished results). It seems likely, therefore, that some other protein factor(s) might be involved in the signal transduction process. To identify such factors, it would be relevant to extend this approach using retrovirus-mediated gene-trap mutagenesis studies.

Germline mutations in the *FLCN* gene have been identified in patients with Birt-Hogg-Dubé (BHD) syndrome, which is an autosomal dominant disorder characterized by hamartomas of skin follicles, spontaneous pneumothorax, and renal tumors [20–22]. Furthermore, *BHD* heterozygous knockout mice were revealed to develop kidney cysts and tumors as they aged, while *BHD* homozygous null mice displayed early embryonic lethality [34,35]. The recent findings, including this report, strongly suggest that folliculin has physical and/or functional interactions with the AMPK-mTOR signaling pathway [17,34,36]. Mutations in several other tumor suppressor genes, such as *LKB1*, *TSC1* and *TSC2* [29,37], have also been shown to lead to dysregulation of AMPK-mTOR signaling and to the development of other hamartomatous syndromes. Our present findings that folliculin is involved in the induction of apoptosis might shed some light on the physiological roles of *BHD/FLCN* and other related tumor suppressor genes. We are currently establishing *Mapo1* knockout mice to analyze the possible roles of the gene in the suppression of tumor predisposition resulting from environmental stresses.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

Acknowledgments

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Genetic susceptibility to lung cancer

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1. ABSTRACT

Lung cancer is a highly environmental disease, but cancer researchers have long been interested in investigating genetic susceptibility to lung cancer. This paper is a historical review and provides updated perspectives on lung cancer susceptibility research. The recent introduction of easier genotyping methods and the availability of an almost complete human genome database facilitated the association study to thousands of cases and controls for millions of genetic markers. Discoveries in the field of behavior genetics, that is, the genetic aspects of smoking behavior and nicotine addiction, unexpectedly indicated that polymorphisms in the human central nervous system play an important role in eventually leading to lung cancer. These findings were achieved by using comprehensive approaches, such as a genome, transcriptome, or proteome approach, and the studies were often conducted without a hypothesis. Another–omics approach, the "adductome" or "exposome" approach to how life style information can be integrated into the framework of genetic association studies, has recently emerged. These new paradigms will influence the area of lung cancer risk evaluation in genome cohort studies.

2. INTRODUCTION

The genetic aspects of the etiology of lung cancer have been considered less important, because the urban vs. country, male vs. female, and smoker vs. non-smoker differences in its incidence, twin studies, and immigration studies taken together have indicated that the etiology of lung cancer is largely environmental (1). On the other hand, in animal carcinogenesis studies Kouri *et al.* found that the inducibility of aryl hydrocarbon hydroxylase is associated with susceptibility to induction of lung cancer by 3-methylcholanthrene (2), and in 1984, Ayesh and Idle published a study showing that debrisoquine hydroxylase activity was higher in lung cancer patients than in healthy controls. Debrisoquine was a popular anti-hypertensive drug used in Europe at the time (3). The work by Ayesh and Idle pioneered the field of pharmacogenetics, and carcinogenesis researchers started to investigate genetic susceptibility to environmental cancers to test the hypothesis that the cancer susceptibility of people exposed to certain environmental carcinogens varies with their genetic capacity to handle (activate, detoxify) xenobiotics. The discipline of molecular epidemiology of human cancer combined with dosimetry studies to assess individual

exposure to particular environmental carcinogens burgeoned (4-7).

3. CANDIDATE GENES FOR INCREASED LUNG CANCER SUSCEPTIBILITY

3.1. Polymorphisms in carcinogen-activating enzymes and lung cancer susceptibility

After Ayesh's work on individual differences in metabolizing debrisoquine (debrisoquine hydroxylase), genes responsible for xenobiotic metabolism were cloned, and a nomenclature system was devised according to the new cDNA sequences that were being isolated on an almost daily basis during that period.

Individual differences in the cytochrome P450 family of genes, the most important family of genes encoding enzymes that are responsible for the metabolism or activation of various environmental chemicals in cells, were extensively investigated by using the polymorphisms of these genes, which had just started to be discovered and to accumulate. An enzyme that activates polyaromatic hydrocarbons (PAHs) to their nucleophilic forms, *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1), was studied most extensively in regard to lung cancer susceptibility, especially tobacco-related lung cancer susceptibility. Kawajiri first reported identification of a polymorphism near the *CYP1A1* locus (actually an Msp I polymorphism in the 3' flanking region; m1, 4646903) and its relation to lung cancer susceptibility (8). Since the polymorphism first proposed was in the non-coding area of the gene, the mechanistic rationale was obscure. Subsequently, however, a more convincing polymorphism, an amino acid substitution polymorphism (m2, Ile462Val in exon 7, the substrate binding region, A2455G; rs1048943 according to current nomenclature) linked to the Msp I polymorphism (m1, rs4646903), was discovered (9). Follow-up studies of Japanese, Okinawan, and Brazilian subjects, in addition to Caucasian subjects, were conducted (10-14), and the association between the single nucleotide polymorphism known as reference SNP rs1048943 and tobacco-related cancer susceptibility was replicated in some but not all of the studies. Actually, the overall results of the follow-up studies on the association between lung cancer and rs1048943 were less convincing than reported earlier, because the associations were not always replicated. To begin with, since the prevalence of the minor, presumably high-risk allele of CYP1A1 was found to be very low in non-Asians, very large populations were required to obtain a modest increase in odds ratio in non-Asian populations, for example, in Scandinavians. Next, the correlation between the CYP1A1 polymorphism and lung cancer seemed to exist only in regard to tobacco-related lung cancer according to the hypothesis that tobacco-related carcinogens induce tobacco-related cancers via this allele that has higher carcinogen-activating capacity. Obviously, rigorous study designs that integrate the histological type of the lung cancer in the case and smoking history and other confounding factors of cases and controls require much more labor and time, especially in populations where the frequency of the high- risk allele is lower. It was only later that a pooled analysis revealed the association between the *CYP1A1* polymorphism and lung cancer, both squamous cell carcinoma and adenocarcinoma, in a Caucasian population (15).

Another group of Japanese researchers investigated the relationship between *CYP2E1* polymorphisms and lung cancer susceptibility in a Japanese population (16, 17), and *CYP2E1* polymorphisms were subsequently investigated in various populations (18-20). Since *CYP2E1* is involved in the activation and metabolism of alcohols and nitrosamines (presumed carcinogens in food in addition to tobacco smoke), the research was directed at gastrointestinal cancers. An interaction between CYP2E1 polymorphism and dietary meat and vegetable intake was reported in colorectal cancer (21). On the other hand, in the field of tobacco-related lung carcinogenesis, Kato *et al.* measured 7-methyl-dGMP (deoxyguanosine monophosphate), N-nitrosamines, and PAH-dGMP adducts in human autopsy lungs and correlated the amounts of various adducts derived from tobacco smoke, environmental tobacco smoke, and exposure to products of combustion of substances other than tobacco, e.g., products of combustion in occupational settings and fuel combustion products) with genotypes of *CYP2E1*, *CYP2D6*, *CYP1A1*, and *GSTM1* (22). Their study revealed that higher 7-methyl-dGMP adduct levels were associated with the presence of *CYP2E1* minor alleles.

CYP2A6 is thought to be responsible for nitrosamine and nicotine metabolism and has been another important target of research on CYP family gene polymorphisms (23). A group led by Kamataki investigated hundreds of lung cancers in Japan and discovered that a deletion- type polymorphism at the *CYP2A6* locus reduces the risk of lung cancer (24, 25). *CYP2A6* is located adjacent to its pseudogene, and identification of the deletion is sometimes technically demanding, but introducing this kind of genotyping by means of SmartAmp™ technology into clinical settings, such as outpatient clinics with a smoking cessation program, is now being widely considered (26, 27).

3.2. Polymorphisms of genes responsible for detoxification and conjugation of carcinogen metabolites

Glutathione-S-transferases (GSTs) are the most extensively studied class of enzymes that are characterized by individual differences in detoxifying activity. The deletion type polymorphism (null type) was well known long (28-30) before extensive copy number analysis of the entire human genome revealed the presence of tremendous insertion/deletion polymorphisms and copy number variations in the human genome (31). Combinations of polymorphisms of genes responsible for detoxification of carcinogens and for activation of procarcinogens were used to evaluate the cancer risk of individuals. Information on life-style factors that increase cancer proneness (e.g., smoking) or that some evidence indicates may be cancer-protective (e.g., consumption of green tea) was also included in multifactorial analyses of the gene-environmental interactions of the polymorphisms of the genes described above (12, 32, 33).

3.3. Repair-gene polymorphisms and lung cancer susceptibility

The canonical "carcinogen and consequent mutation theory" states that the ultimate carcinogen binds to DNA, thereby producing a DNA adduct that induces a base-pair replacement (mutation) in the next cycle of DNA replication, unless the DNA adduct is removed or the cell dies. Humans have a huge redundant system to repair such DNA damage (34, 35).

A classical example of a link between a defect in repair genes and human cancer is xeroderma pigmentosum, which comprises at least 9 different diseases, each with its own responsible gene (36-38). However, the most extensively studied gene from the standpoint of human genetic susceptibility to cancer is *hOGG1*, a base excision repair gene that removes 8-oxo-guanine (7,8-dihydro-8-oxoguanine) opposite cytosines. Soon after the isolation and characterization of *hOGG1* by several groups (39-42), a nonsynonymous variation Ser326Cys (rs1052133) was identified (43), and the first case-control association study of human lung cancer was performed (44). It was a small study, and many studies, pooled studies, and meta-analyses of different populations followed (45-50). One report proposed a mechanistic basis for the contribution of the minor variant 326C to carcinogenesis (51). Since the original hypothesis was based on the reasoning that oxygen free radicals damage DNA, which results in mutations if DNA repair is inadequate, and the mutations in turn lead to a predisposition to cancer, especially in the organs often exposed to damage by reactive oxygen species, such as the lungs of smokers. The inference would be that tobacco-related cancer is more significantly correlated with the polymorphism than non-tobacco-related cancer. Actually, the first paper to describe an association between OGG1 and lung cancer reported finding a positive correlation between *OGG1* polymorphism and squamous cell carcinoma of the lung (44). However, a meta-analysis of studies in which there were high numbers of cases of adenocarcinoma of the lung indicated a possible association between the *OGG1* Ser/Cys polymorphism and adenocarcinoma, too (50).

Another issue raised by many researchers is ethnic differences, e.g., with respect to the *CYP1A1* Ile426Val polymorphism (rs1048943). A meta-analysis of a total of 6375 cases and 6406 controls revealed an association between the *CYP1A1* Ile426Val polymorphism (rs1048943) and lung cancer in Asians alone (45). The meta-analysis did not mention any interaction with the histological subtypes of the lung cancers, whose subtyping is sometimes very subjective and affected by severe inter-observer bias despite widespread standardization as in the WHO classification, and not all of the studies included in the meta-analysis contained complete smoking histories. The conclusion of the meta-analysis that "careful matching should be considered in future larger genetic association studies that include multiple ethnic groups" is quite obvious.

Another polymorphism in repair genes that has been studied extensively in an epidemiological framework is xeroderma pigmentosum group D (XPD) or excision repair cross-complementing rodent repair deficiency, complementation group 2 (ERCC2). XPD, i.e., ERCC2, is a well- characterized DNA helicase that is required for nucleotide excision repair of bulky DNA lesions (larger than adducts like 8-oxoguanine). A relatively rare missense variant, Lys751Gln (persons with the Lys/Lys genotype are less able to repair DNA), was discovered (52), and although no association with lung cancer was found in an earlier study (53), a significant difference between cases and controls was found in a Chinese study (54, 55). In contrast to the first report by Lunn *et al.*, the Gln allele was found to be a high-risk allele in another Chinese lung cancer case-control set (56). However, the Asp312Asn polymorphism did not appear to affect DNA repair in the first study (52), but the results of the next study suggested a negative effect on DNA repair capacity in the homozygous Asn/Asn genotype (57). A meta-analysis of 9 papers in 2005 found no clear correlation between *XPD* polymorphism and lung cancer (58).

In 2008, an international lung cancer consortium analyzed the previous papers on 12 repair genes and their 18 polymorphisms and lung cancer susceptibility, and the consortium concluded that *OGG1* Ser326Cys, *TP53* Arg72Pro, *XRCC3* Thr241Met, and *XPD* Lys751Gln were weakly associated with increased lung cancer susceptibility (47). The consortium suggested that further data pooling and a genome-wide association study approach were needed.

3.4. Oncogene and suppressor gene polymorphisms, and other genetic and epigenetic variations to modify lung cancer susceptibility

Some of the inter-individual variation of the genes tightly involved with human carcinogenesis has also been a topic enthusiastically investigated. The champion of this category would be an Arginine-Proline polymorphism in the exon 4 of TP53 (Arg72Pro, rs1042522). A comprehensive meta-analysis consisting of 302 case-control studies of cancers of all the organs suggested the contribution of this polymorphism depends on anatomic site of cancers (59). A meta-analysis consisting of 7495 lung cancer cases and 8362 controls based on 23 studies concluded that Pro allele is a low penetrant risk factor for developing lung cancer (60). Among the oncogenes, K-RAS is often mutated in lung cancer (61) and has been suggested to be responsible for genetic susceptibility to mouse pulmonary adenoma (62). In human lung cancer, the SNP around the *K-RAS* locus was not known to be associated to lung cancer susceptibility, so far.

Recently, frequent somatic methylatin of LKB1 in human lung cancer was found especially in Caucasian lung cancer and it is elusive this phenomenon is related to some genetic predispositions (63). Some of the germline variants in methyl-group metabolism genes are reported to be associated with somatic methylation profile of several genes including LKB1 in lung cancers (64).

Involvement of the genes which often somatically mutated or methylated in lung cancers are expected to explain some of lung cancer risk (65). However, the epigenetic change passable to the next generation (epimutation) was not found in lung cancer (66).

In addition to the SNPs in the genes mentioned above, any SNPs in the loci of the genes associated with human carcinogenesis have great potential in terms of possible contributions of efficient identification of lung cancer risk. Actually genome-wide association study disclosed an association of TP63 polymorphism and adenocarcinoma of the lung (67).

4. THE ERA OF THE GENOME-WIDE ASSOCIATION STUDY (GWAS)

The first GWAS on lung cancer susceptibility was published in 2007 (68). DNA from 100 cases and 100 matched controls was used, presumably to minimize cost. The experiments were performed on the Affymetrix (Santa Clara, CA) GeneChip platform by applying the human mapping 50K Xba240 and Hind240 arrays according to the manufacturer's protocols (68). The study identified 38 SNPs that might be associated with increased lung cancer susceptibiltiy. By current standards, the small numbers of samples used to conduct the investigation and the economical strategy of using pooled DNAs were very modest. The authors were aware that the 100K chip did not contain some of the SNPs that had already been reported to be associated with lung cancer. Several large-scale studies were published in the spring of the following year (47, 69-72), and they had investigated more than 300,000 SNPs (Illumina HumanHap300 v1.1 BeadChips) in case-control sets of more than 1000 pairs. Moreover, all of the studies included replication study sets that consisted of additional thousands of case-controls. Amazingly and interestingly, the loci indentified encoded nicotinic acetylcholine receptors (chromosome 15q25.1) that had been thought to be related to smoking behaviors. The rs10151730, rs8034191, and rs16969968 (Asp398Arg substitution polymorphism of *CHRNA5*) polymorphisms were significantly associated with lung cancer in people of European descent. A study of Icelanders (72) revealed the important finding that the rs1015730 locus was related to nicotine dependence, lung cancer, and peripheral arterial diseases. Late the same year, another locus was identified, based on an additional 3000 or so cases-controls (73). This study corroborated the previous studies that showed an association with chromosome 15q25 loci, especially with the rs1015730 locus, identified as rs402710 and rs2736100, which the investigators claimed, were independent, and the nearby genes were *TERT* and *CLPTM1L*, respectively. Another study, published at almost at the same time, identified two loci at 6p21 and an additional polymorphism in the *CLPTM1L* gene locus (intron 13, rs401681) (74).

The presumed risk-alleles above were tested in lung cancer cases in which there was a family history of lung cancer (75). At the same time, the group that reported finding an association between *CHRNA5-A3* (rs1051730 and rs803419) and lung cancer (69) discovered that these genotypes were also associated with both nicotine dependence and lung cancer causation (76).

Many replication studies on the relationship between CHRNA5-A3 loci, nicotine dependence, and lung cancer were published in the next several years. Amos *et al.* reported associations between multiple loci (including rs169698) and increased risk of lung cancer in African Americans, instead of smoking (behavioral) phenotype (77). Wu *et al.* identified three novel SNPs (rs2036534C>T, rs667282C>T, rs12910984G>A, and rs6495309T>C) that are common in Asians and are related to smoking behavior and increased lung cancer risk in a Chinese population (78). Shiraishi *et al.* even demonstrated a possible contribution of three SNPs around the *CHRNA5* locus to lung cancer risk in Japanese, whose prevalence of minor alleles at the three loci (rs8034190, rs16969968 and rs1051730) is very low (79).

The above genome-wide studies and a later one recruited thousands of case-controls in several populations, but the overall contributions of the genotypes accounted for only 1% of the excess familial risk of lung cancer (80). The authors of the later study claimed that a larger sample of DNAs from a series of lung cancer cases and controls with records of smoking behavior would be necessary in addition to the currently or previously collected DNAs in order to identify genes associated with increased risk of lung cancer (71)!

GWASs conducted in the last several years have demonstrated that new technology enables hundreds of SNPs to be processed in thousands of cases without a set of working hypotheses (81). The susceptibility alleles that have been discovered with the new technology by GWASs are common (present in 10% or more of Caucasians). Each allele makes a small contribution, and the odds ratios calculated for the high-risk genotypes are usually less than 1.3. The pursuit of lung cancer susceptibility genes by GWASs has been same as the pursuit of susceptibility genes for other diseases by GWASs.

However, probably because lung cancer is a very environmental disease, as previously mentioned, based on current theoretical inferences, only three genes are suspected of being lung cancer susceptibility genes, far fewer than in more genetic cancers (prostate cancer, for example, in which the number is estimated to be 30). In view of the largely environmental nature of lung cancer and the fact that there are expected to be fewer genetic components than in other cancers, an extra twist in the strategy will be necessary to zero in on lung cancer susceptibility genes. For example, the next association study must include stratification of lung cancers according to smoking history in addition to ethnicity and histological type (82, 83). On the other hand, smoking itself is now considered a highly genetically controlled behavior. The genetic aspect of smoking behavior is addressed in the next section.

5. SMOKING BEHAVIOR AND LUNG CANCER SUSCEPTIBILITY

Smoking by male members of the same family is common in many cultures, especially in many traditionally

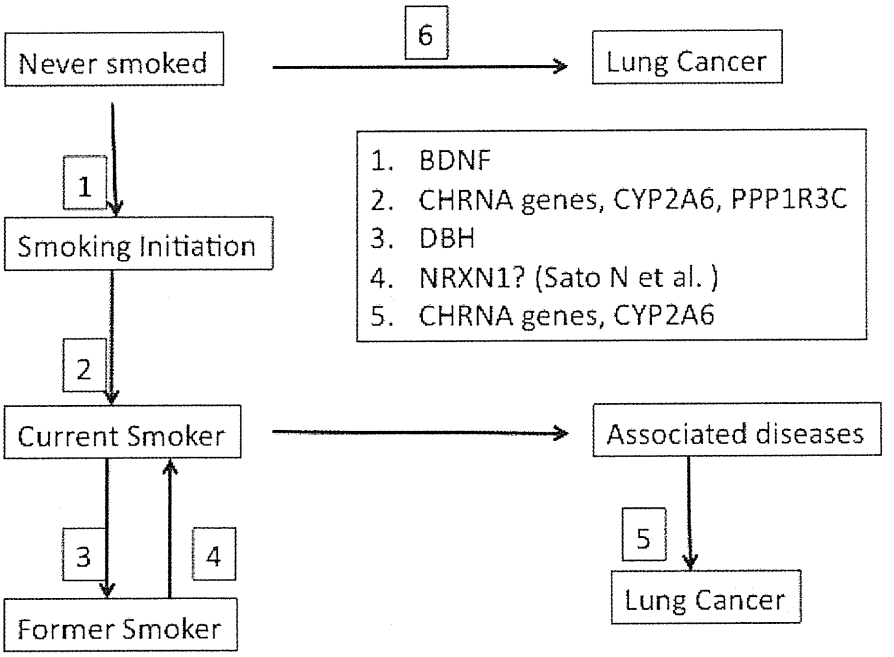

Figure 1. Presumed steps and genetic influences in the path from smoking to lung cancer. (Modified from (101). Amos CI, Spitz MR, Cinciripini P: Chipping away at the genetics of smoking behavior.) 1. Smoking initiation, 2. Smoking dosage, 3. Smoking cessation, 4. Difficulty quitting smoking, 5. Smoking dependence (cigarettes smoked per day), 6. A missing link in the mechanism of lung carcinogenesis in never smokers.

male chauvinistic cultures. Actually, two papers, one published in1963 and the other in 2003, stated that familial clustering of lung cancer can be explained by familial clustering of smokers (84, 85). However, the results of a recent simulation study did not support clustering of smokers in families as a cause of familial cases of lung cancer (86).

On the other hand, the heritability of substance dependence, including dependence on tobacco (nicotine), has been investigated in many populations (87). Nicotine dependence is now recognized as a disease according to the International Statistical Classification of Disease and Related Health Problems (ICD) 10 and is treated in smoking-cessation clinics, and various genes have been assessed as candidates for genes responsible for nicotine dependence (88). Candidate genes related to] dopaminergic pathways have attracted the attention of many investigators (89), but a genome-wide approach was proposed (90) and several ambitious trials have been conducted (91). Saccone *et al.* have found a few chromosomal regions by performing a genome-wide linkage analysis and Bierut identified several other regions by using high-density SNP arrays (92, 93). The results of those studies yielded a very long list of candidates for addiction genes (94). Hundreds of genes responsible for addiction may play a role in tobacco-related carcinogenesis, including in lung cancer. Smoking behavior has traditionally been assessed by means of several different types of questionnaire, and the most-widely used questionnaire is the Fagerström Test for Nicotine Dependence (FTND) (95). Another test, the Tobacco Dependence Screener (TDS), was recently developed. The questions in the TDS regarding each symptom or trait correspond to the criteria in the ICD -10 and Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (96). Many other questionnaires have been devised besides these two, but only a few studies have used more than two of the questionnaires to compare their ability to detect dependence and detect associations with genotypes, and each questionnaire may actually detect different genetic traits related to smoking (92, 97, 98). The Tobacco and Genetics Consortium, which consists of 116 researchers, recently showed that multiple loci are associated with smoking behavior (99), and Liu *et al.* confirmed that a locus on chromosome 15, the promoter region of *CHRNA5*, is a locus responsible for smoking behavior (nicotine addiction) in one of the same cohorts, the Oxford-GlaxoSmithKlein cohort (100). Interestingly, the Consortium identified *BDNF*, which is related to various neurological functions in humans, as an initiation gene, and *DBH*, an important gene related to the dopaminergic pathway, as a continuation (failure to stop smoking) gene. These findings prompted the Consortium to devise a flowchart showing the pathways from the start of smoking to the development of lung cancer (101). (Figure 1) This concept that a genetically determined smoking behavior pattern is the origin of human lung cancer as depicted in Figure 1 is obviously an oversimplification, but it warrants verification in various populations and in various SNPs of the candidate genes.

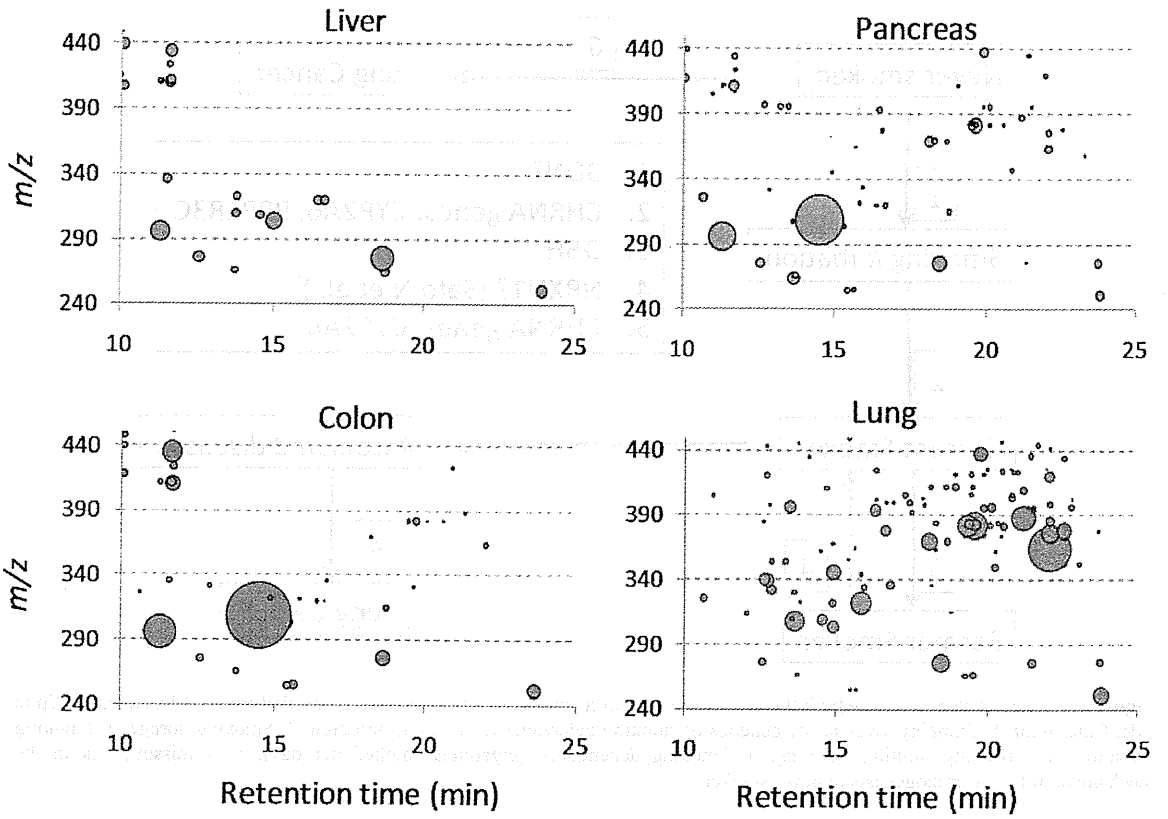

Figure 2. Adductome maps of 4 organs of the same individual. The position of each circle represent the species of the DNA adducts, and the size of each circle indicates the relative quantity of each DNA adduct. The adductome map shows that each organ contains more than one type of DNA adduct. The profile of adducts varies with the organ in the same individual. The profile of the lung adductome map (lower right) shows many kinds of adducts.

6.EXPOSURE ASSESSMENT AND LUNG CANCER SUSCEPTIBILITY

The susceptibility of an individual to lung cancer depends more on the extent to which the individual has been exposed to carcinogens than on the pattern of gene expression in each of the individual's organs (lung, brain, etc.). Estimating how many possible carcinogens are in the body is a challenge, because the tissue samples are usually inadequate, and we do not know exactly what kind of chemicals might be present. The history of painstaking isolation and synthesis of chemicals that cause cancer in animals since Yamagiwa succeeded in inducing skin cancer by painting tar on a rabbit's ear (102) revealed that numerous chemicals that were products of combustion, endogenous oxidation, and generated *in vivo* as byproducts of inflammation can cause cancer. Furthermore, how these carcinogens act on DNA, form covalent bonds with DNA, cause mutations, and induce neoplastic transformation in cells became clear, especially in *in vitro* systems and in experimental animals (103, 104). Moreover, recent studies have shown that a typical mutagenic carcinogen is involved in carcinogenesis via its actions on physiologically important cell machinery that are not necessarily accompanied by mutation induction (105, 106). Thus, the remaining questions are how to validate the formation of these adducts that "carcinogenesis" studies have indicated occurs in the human body and how to evaluate their effects. Several methodologies have been invented to answer these questions, and some have succeeded in detecting causes of human carcinogenesis, i.e., a particular adduct in a particular cancer in a particular setting (4, 107, 108), but since these methods are capable of detecting only a limited numbers of adducts and human body may contain a wide variety of adducts it was hoped that a more efficient method that would detect multiple species of adducts in many samples would be found. The recent progress in the field of bio-measurement has facilitated the simultaneous detection of multiple adducts in the same human tissues (109, 110). DNA adducts have been found to vary with the organ analyzed in the same individual (Figure 2), showing that each organ has a different profile of adducts, probably as a result of exposure to different sets of carcinogens, e.g., the lung to air-bone carcinogens, the colon to food-borne carcinogens, the skin to environmental carcinogens, etc., and organ-specific metabolism. In the adductome map in Figure 2 liquid chromatography retention time is shown on the horizontal axis, and mass spectrometry molecular

DNA species in individual human tissues.

Table 1. Genetic polymorphisms most extensively evaluated for lung cancer susceptibility[1]

Gene name	Rs number	Variation class	Polymorphism	Rationale[2]	References[3]
CYP1A1	rs1048943	SNP	Ile463Val	substrate binding domain	10
CYP2A6		deletion		loss of function	24
CYP2E1	rs2031920	SNP	C/T	promoter site	22
GST-M1		deletion		loss of function	28
OGG1	rs1052133	SNP	Ser326Cys	altered activity	44
XPD	rs13181	SNP	Lys751Gln	repair capacity	46
XRCC3	rs8615339	SNP	Thr241Met	adduct level	46
TP53	rs1042522	SNP	Arg72Pro	E6/ubiquitin-mediated degradation	60

[1]Note: "most extensively investigated" does not mean the greatest contributing risk toward lung cancer occurrence, [2]Assumed rationales. Some of them remains controvertial, [3]Only one reference number is shown here for each gene.

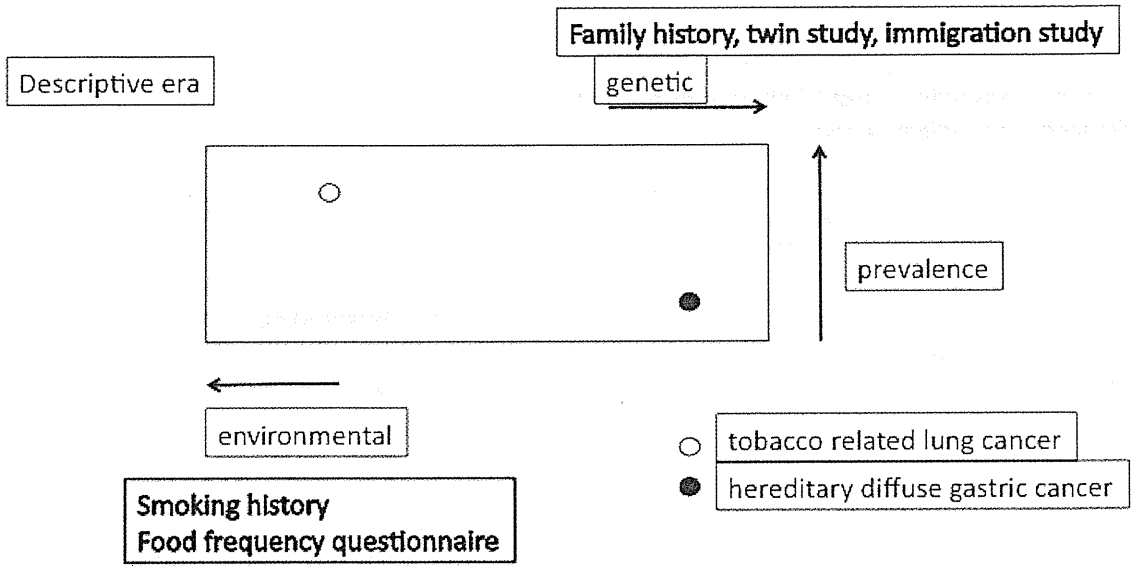

Figure 3. Methodological developments in nature and nurture analyses of cancer susceptibility. The arrows how much environmental and genetic components influence the incidence of diseases. A disease like lung cancer (open circle) would be located in a more environmental position in the spectrum, whereas a late-onset genetic cancer like hereditary diffuse gastric cancer (HDGC) (closed circle) would be located in more genetic position in the spectrum. The prevalence of lung cancer is much higher than that of HDGC. Figure 4 and 5 are the same. Descriptive era: A descriptive history of life style, including smoking history, a nicotine dependence test, and a food-frequency questionnaire were the main methods of estimating exposure. Family history, twin studies, and immigration studies played a great role in assessing genetic elements.

weight per charge on the vertical axis. Only some of the spots have been annotated (109). Complete annotation would reveal the overall exposure status of human organs and promote further quantitative characterization of the modified DNAs, including the mutagenicity of the modified DNAs. Even in this pilot study, differences in numbers and sizes reflecting the approximate amount of the individual adducts can be seen between smokers' lungs and never-smokers' lungs (100). Chou *et al.* applied the adductome approach to larger numbers of cases and succeeded in discovering considerable amounts of lipid peroxidation-induced DNA adducts (110). Lipid peroxidation-induced DNA adducts are derived from omega 3 and omega 6 polyunsaturated fatty acids, which are endogenous in all body tissues. These adducts can be used as a surrogate markers to estimate how much an individual has been exposed and will provide a clue as to how environmental or endogenous mutagens trigger neoplastic transformation in cells. A specific analysis of how the cellular machinery responds to these adducts in the human body andpresumed individual differences in their repair capacity is needed.

6. PERSPECTIVES

As Gazdar provocatively mentioned, lung cancer is mainly associated with smoking behavior and with environmental exposure to tobacco smoke in other words, it is a highly environmental disease. Lung cancers in never-smokers account for 25% of all lung cancers, and the histological type found in never- smokers is very different from the histological types found in smokers] (111). The results of some SNP analyses of lung cancers were not

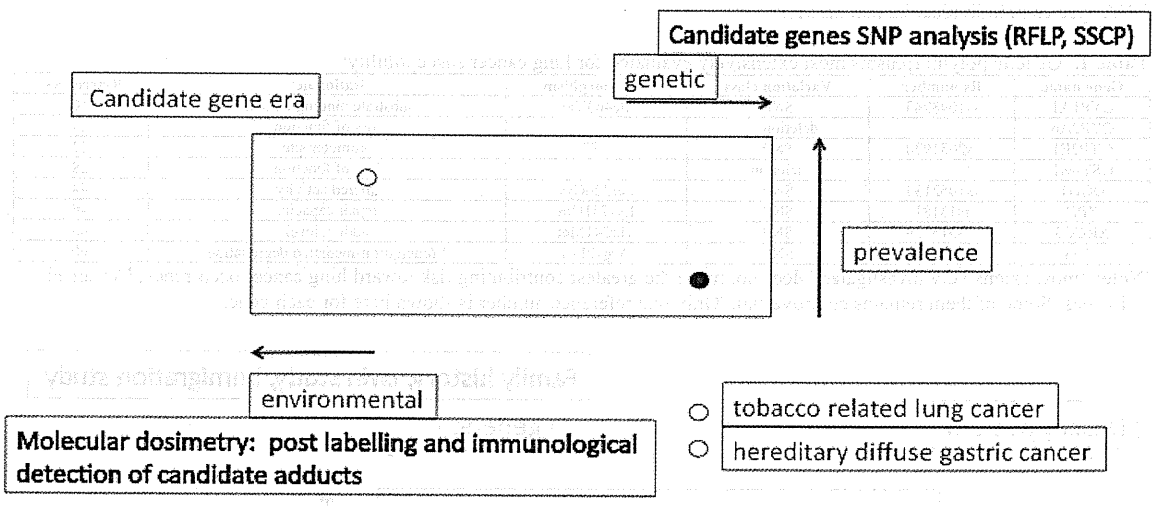

Figure 4. Candidate gene era: Some technical developments in molecular dosimetry, such as the post-labeling method and enzyme-linked immunoorbent assays using antibodies to adducts, were introduced. Genetic polymorphisms, such as restriction fragment length polymorphisms and single-strand conformation polymorphisms, were widely adopted to indentify individual genotypes.

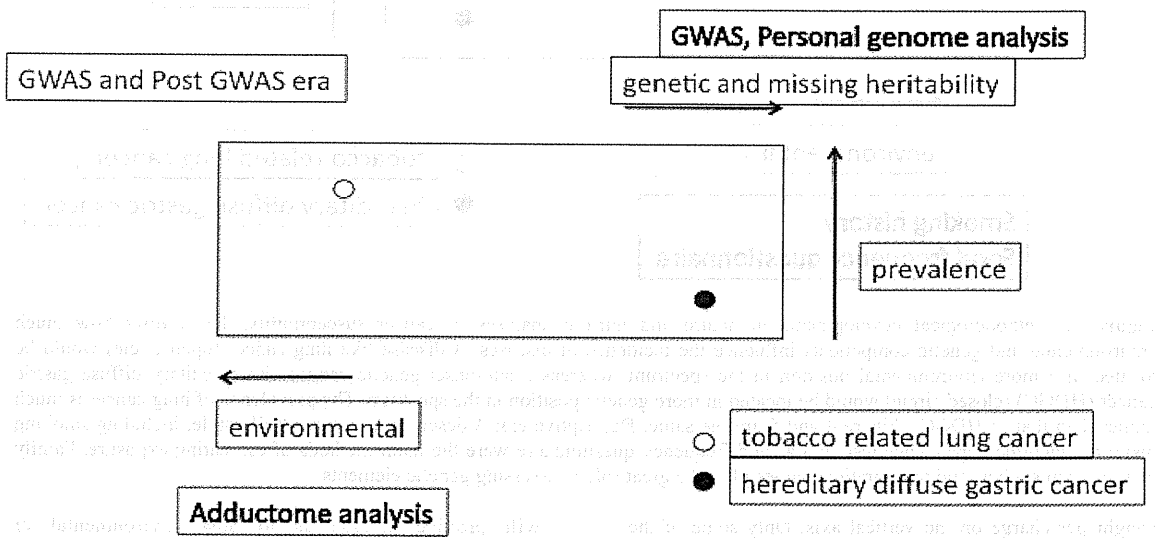

Figure 5. GWAS and post-GWAS era: The GWAS approach and post-GWAS approach (personal genome by next generation sequencing methods) are included among the methodologies aimed at the genetic aspects of lung cancer susceptibility. Adductome analysis emerged as a comprehensive method of detecting multiple modified

replicated in a subsequent meta-analysis that included never-smokers' lung cancers (112), probably because the study was "underpowered" (only thousands of cases!). Copy number variants have never been fully estimated in lung cancer susceptibility studies. Genomic analyses will soon become more economical, and the era of personal genome analysis is at hand. Obviously, careful and comprehensive studies on genotype-phenotype associations (113) need to be performed in humans. When we review the previous progress in methodologies for analyzing both the nature and nurture aspects of cancer susceptibility (Figure 3-5), we cannot avoid expecting a future in which "personal genome x personal adductome" information will provide a definitive assessment of each individual's risk of lung cancer.

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