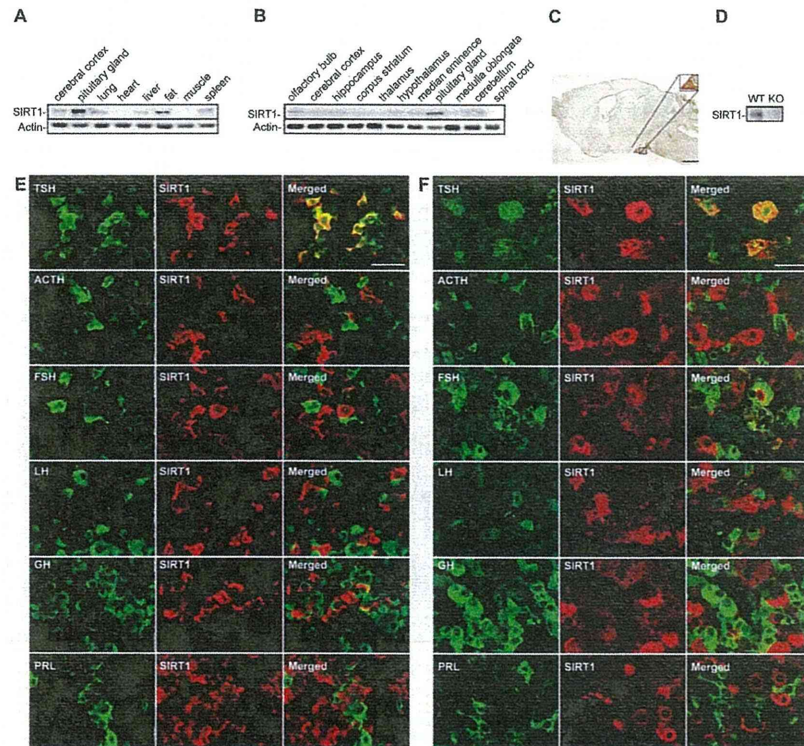

Figure 1. Effect of SIRT1 on hormone secretion. (A, B) Western blot analysis of SIRT1 in the systemic organs (A) and nerve tissues (B) of mice. (C) Immunohistochemical staining of SIRT1 in the rat brain. The upper right panel shows the anterior pituitary area at higher magnification. Scale bar, 1 cm. (D) SIRT1 immunoreactivity is absent in the pituitary gland of SIRT1 KO mouse. (E, F) Double immunostaining with SIRT1 (red) and pituitary hormones (green) in the pituitary gland of a 12-weeks old mouse (E) and a 64-years old human (F). Scale bar, 100 μm.
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kinase [28]. Thus we used the catalytically inactive form (H355A) of SIRT1 [29,30], to screen for binding partners. The Flag-tagged SIRT1 and SIRT1 (H355A) constructs were expressed in HEK293T cells and immunoprecipitated with an anti-Flag antibody. The immunoprecipitates were digested and then subjected to a nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS) system [31]. Proteins identified as candidates of SIRT1 binding partners included phosphatidylinositol-4-phosphate 5-kinase (PIP5Ks) (Fig. 3A). The enzymes PIP5Ks (PIP5Kα, PIP5Kβ, and PIP5Kγ) are enzymes that catalyze the synthesis of $PI(4,5)P_2$ mainly through the cellular route [32]. We focused on PIP5Ks since PIP5Kγ is reported to play an important role in the exocytosis of large dense-core vesicles by inducing the synthesis of $PI(4,5)P_2$ in endocrine cells [33].

The common regions of all PIP5Ks were pulled down with SIRT1 in the fist screening with LC-MS/MS. To examine if SIRT1 more specially interacts with PIP5Kγ than with the other PIP5Ks, we performed immunoprecipitation analysis against endogenous proteins with antibodies to SIRT1 and to PIP5Ks. Amongst the PIP5Ks examined, PIP5Kγ was selectively immunoprecipitated with SIRT1 (Fig. 3B). Further investigation with deletion mutants of PIP5Kγ revealed that the interaction between SIRT1 and PIP5Kγ was mediated through a region of the kinase core domain [34] (amino acid residues 247—281) of PIP5Kγ (Fig. 3C).

SIRT1-mediated deacetylation of PIP5Kγ

The binding of SIRT1 with PIP5Kγ suggests that SIRT1 deacetylates acetylated PIP5Kγ. Treatment with nicotinamide

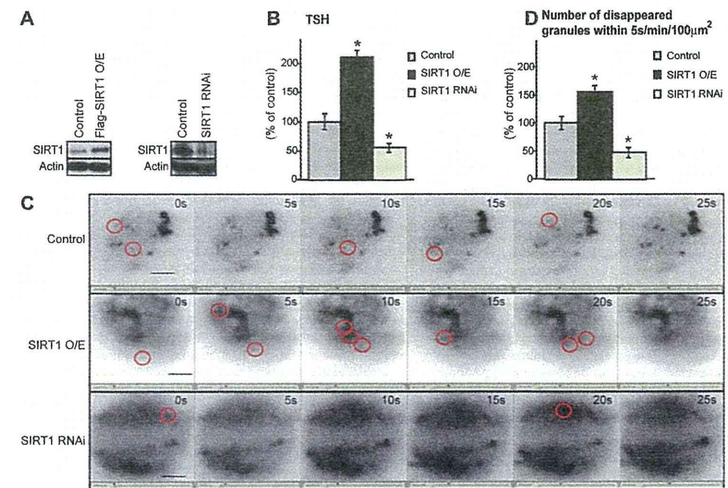

Figure 2. SIRT1 upregulates exocytosis of TSH. (A) Western blot analysis of SIRT1 in primary pituitary cells and in cells overexpressing (O/E) SIRT1 (left panel) and SIRT1 RNAi cells (right panel) levels. (B) Amounts of TSH secreted by SIRT1-O/E and SIRT1-RNAi pituitary cells over 24h ($n = 10$). Statistical analyses were performed using Fisher's PLSD test. *$P<0.05$ versus control. (C) A TIRF image of an anterior pituitary cell. Several GFP-TSH granules that disappeared within 5 s are circled in red. Scale bar, 20 μm. (D) Number of expelled TSH granules were morphologically identified using the TIRF images ($n = 5$). Statistical analyses were performed using Fisher's PLSD test. *$P<0.05$ versus control. Values represent mean±SEM (% of control: B and D).
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(NAM), a SIRT1 inhibitor [35], increased the lysine acetylation of PIP5Kγ, but did not alter the acetylation levels of PIP5Kα and PIP5Kβ (Fig. 4A).

The SIRT1-binding site of PIP5Kγ has 2 lysine residues (residues 265 and 268; shaded in Fig. 4B) besides the 5 identical lysine residues conserved in all the 3 PIP5Ks (residues 255, 260, 266, 270 and 276) (Fig. 4B) [34]. Within light of the preferential interaction between SIRT1 and PIP5Kγ, we hypothesized that SIRT1 mainly deacetylates lysines 265 and/or 268 of PIP5Kγ. To test this hypothesis, we constructed a PIP5Kγ plasmid in which 2 lysine residues were substituted with arginine residues (K265/268R). As expected, the K265/268R mutant was not further acetylated, regardless of treatment with NAM (Fig. 4C). This indicates that SIRT1 deacetylates the residues 265 and/or 268 of PIP5Kγ. To prove that SIRT1 directly deacetylates PIP5Kγ, we performed an *in vitro* deacetylase activity assay. PIP5Kγ deacetylation occurred upon the addition of recombinant wild-type SIRT1 but not the inactive form of SIRT1 (H355A) (Fig. 4D). In contrast, PIP5Kα and PIP5Kβ were not deacetylated by SIRT1 (Fig. 4D). Taken together, our findings indicate that SIRT1 selectively deacetylates PIP5Kγ among all the PIP5Ks.

We next asked whether the acetylation state affects the enzymatic activity of PIP5Kγ. We measured the *in vitro* kinase activity of PIP5Kγ with some modifications of the assay system of previous reports [36,37]. We preincubated PIP5Kγ with recombinant wild-type SIRT1 or the inactive form of SIRT1 (H355A) for 3 h at 30°C, and then examined the activity of PIP5Kγ. The production of PIP_2 was significantly increased when PIP5Kγ was deacetylated with wild-type SIRT1, whereas no increase occured when PIP5Kγ was incubated with the inactive SIRT1 (H355A) (Fig. 4E). These results indicate that SIRT1 deacetylates PIP5Kγ, thereby increasing the latter's activity. Consistent with this, PIP_2 production was significantly decreased in HEK293T cells treated with SIRT1-RNAi (Fig. 4F and 4G). Our findings demonstrate that SIRT1 increases the kinase activity of PIP5Kγ by deacetylating this kinase.

Involvement of PIP5Kγ in SIRT1-mediated TSH Secretion

PIP5Kγ is the major $PI(4,5)P_2$ synthesizing enzyme in the brain [38]. In addition, PIP5Kγ KO mice show defects in vesicle trafficking [33]. We examined whether PIP5Kγ was expressed and physically interacted with SIRT1 in the pituitary gland, before testing whether PIP5Kγ is involved in the SIRT1-mediated TSH secretion in pituitary cells. PIP5Kγ is expressed in pituitary gland in addition to other regions of the mouse brain (Fig. 5A). In the pituitary gland, SIRT1 and PIP5Kγ were detected in the cytosolic fraction (Fig. 5B). Further, they colocalized in the cytoplasm of many cells in the anterior pituitary gland (Fig. 5C). These two proteins were coimmunoprecipitated with each other from pituitary cytosolic lysates by antibodies specific for each protein (Fig. 5D). Thus, SIRT1 and PIP5Kγ are physically associated in pituitary cells.

To determine whether PIP5Kγ is involved in SIRT1-mediated TSH secretion in pituitary cells, we knocked down PIP5Kγ in

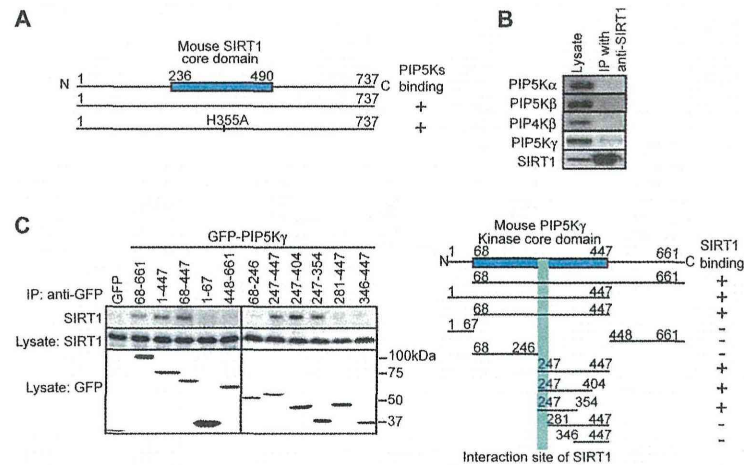

Figure 3. Identification of PIP5Kγ as a SIRT1-binding protein. (A) PIP5Ks were identified as SIRT1-associated protein by immunoprecipitation with anti-FLAG antibodies from HEK293T cells expressing FLAG-SIRT1. A full-length SIRT1 and inactive form of SIRT1 (H355A) interacted with PIP5K (+). (B) Immunoprecipitation analysis of endogenous SIRT1 and endogenous PIP5Kα, PIP5Kβ, PIP5Kγ, or PIP4Kβ in HEK293T cells. (C) Mapping the *interaction interface of SIRT1 with PIP5Kγ. A schematic representation of the SIRT1 interaction site (right panel; positive (+), negative (−)).*
doi:10.1371/journal.pone.0011755.g003

SIRT1-overexpressing pituitary cells. The specificities of RNAi against PIP5Ks were examined; RNAi against each PIP5K showed the specific knockdown effect to each target (Fig. 5E). We confirmed that PIP_2 production was significantly decreased in the PIP5K-RNAi knockdown pituitary cells (Fig. 5F). Basal-level TSH secretion was reduced by the knockdown of PIP5Kγ, whereas neither PIP5Kα-RNAi nor PIP5Kβ-RNAi knockdown showed such an effect (Fig. 5G). Strikingly, the knockdown of PIP5Kγ completely abolished the effect of SIRT1 overexpression on the enhancement of TSH secretion (Fig. 5H). This result demonstrates that SIRT1 and PIP5Kγ work in the same pathway, and supports our concept that SIRT1 regulates TSH secretion via the deacetylation of PIP5Kγ.

SIRT1 KO mice show high acetylated-PIP5Kγ, low PIP_2 and low TSH Secretion

To confirm that SIRT1 regulates TSH secretion *in vivo* via modulating PIP5Kγ acetylation level and its lipid kinase activity, we investigated SIRT1 KO mice. First, we examined the level of acetylated PIP5Kγ in the pituitary gland of SIRT1 KO mice. The level of acetylated PIP5Kγ was higher in SIRT1 KO mice than that in wild-type (WT) mice (Fig. 6A). The PIP_2 levels in the brain were lower in the SIRT1 KO mice than in the WT mice (Fig. 6B).

SIRT1 KO mice have smaller pituitary than wild-type mice [39]. Thus, we examined the number of TSHβ cells in SIRT1 KO mice, before investigating the TSH release. Unexpectedly, the number of TSHβ cells was higher in the pituitary glands of SIRT1 KO when compared to normal (Fig. 6C). Consistent with this finding, the total TSH protein content in SIRT1 KO pituitary was significantly elevated (Fig. 6D). Despite the increased pituitary TSH contents, the amount of secreted TSH from SIRT1 KO pituitary glands was significantly lower than that from WT pituitary glands (Fig. 6E). The difference was more remarkable when the plasma TSH level was normalized with the number of pituitary TSHβ cells (Fig. 6F). There was no difference in the TSH mRNA levels in the thyrotropes of SIRT1 KO and WT mice (Fig. 6G). Taken together, these findings support our model that SIRT1 regulates TSH release through modulating PIP5Kγ activity (Fig. 7).

Discussion

In the present study, we showed that SIRT1 is abundantly expressed in TSH-producing cells in the anterior pituitary gland. Hypothalamic–pituitary–thyroid (HPT) axis regulates energy expenditure, oxygen consumption, and fuel metabolism [40]. Thyroid hormones (triiodothyronine [T3] and thyroxine [T4]) negatively regulate the HPT axis [41,42], and influence adipose tissue metabolism and cholesterol homeostasis [43]. In addition, the combination of serum TSH and tissue insulin sensitivity has important effects on serum lipid parameters in type 2 diabetes [44]. SIRT1 is also involved in insulin sensitivity and lipid metabolism [5]. Under fasting conditions, SIRT1 has a negative effect on insulin sensitivity [45]. Under fed conditions, activation of SIRT1 improves insulin sensitivity [46,47]. Thus it is reasonable to speculate that the SIRT1-PIP5Kγ pathway described in this study represents a key event in the process of energy metabolism.

The longevity response to calorie restriction (CR) is actively regulated by nutrient-sensing pathways involving the kinase target of rapamycin (TOR) [48], AMP kinase [49,50] and insulin/insulin-like growth factor (IGF-1) signalling [51] in lower organisms and mice. Sirtuins is also thought to be linked to CR

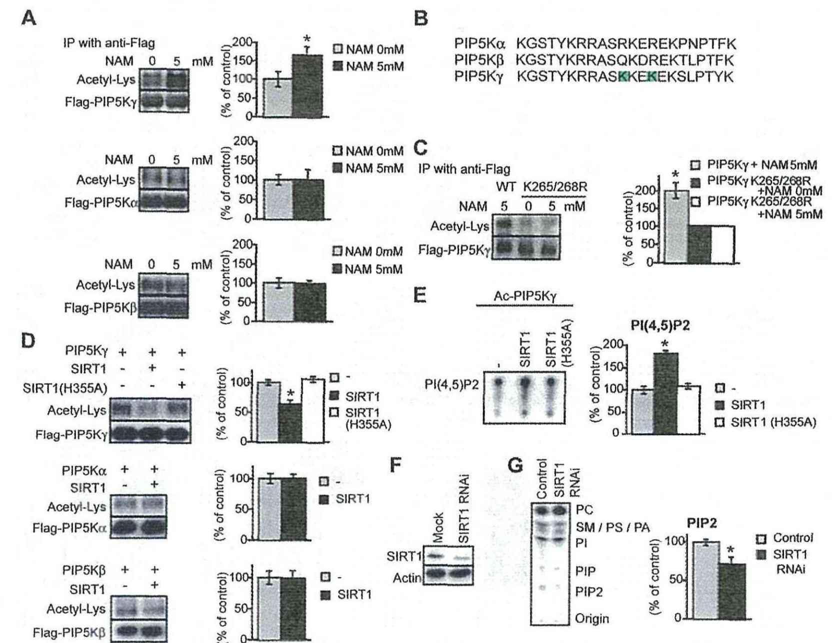

Figure 4. Activation of PIP5Kγ by deacetylation. *(A, C) Effects of NAM on the acetylation of PIP5Ks (A) and PIP5Kγ K265/268R (C). The level of* acetylated-lysine/PIP5K level was determined. Statistical analyses were performed using Student's *t* test ($n=3$). $^*P<0.05$ versus the non-NAM-treated group. (B) Sequence alignment of PIP5Ks. PIP5Kα (amino acids 205–226), PIP5Kβ (246–267), and PIP5Kγ (255–276) are shown. The light blue columns indicate candidates for deacetylation site by SIRT1. (D) *In vitro* deacetylation of PIP5Ks by SIRT1. Each Flag-PIP5Ks were purified from HEK293T cells after treatment with 5 mM NAM and was incubated with full-length recombinant SIRT1 or SIRT1 (H355A) in the presence of NAD^+ for 3 h at 30°C. The acetylated-lysine/PIP5K level was determined. Statistical analyses were performed using Fisher's PLSD test or Student's *t* test ($n=3$). $^*P<0.05$ versus control. (E) TLC analysis of $[^{32}P]PI(4,5)P_2$ produced by acetylated PIP5Kγ incubated with recombinant SIRT1 or SIRT1 (H355A). The $PI(4,5)P_2$ level was measured using the ImageJ software ($n=3$). Statistical analyses were performed using Fisher's PLSD test. $^*P<0.05$ versus control. (F) Western blot analysis of SIRT1 in HEK293 cells and SIRT1 RNAi cells. Actin was used as the control. (G) TLC analysis of $[^{32}P]PIP_2$ that was extracted from SIRT1 KD HEK 293T cells metabolically labelled (for 24 h) with $[^{32}P]$orthophosphate. The PIP_2 level was corrected for the PC level ($n=5$). PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; and PIP, phosphatidylinositol phosphate. Values represent mean±SEM (A–D and F).
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and autophagy to prolong lifespan with TOR pathway [52]. Resveratrol, an activator of sirtuins, antagonizes the mTOR/S6K pathway [53]. In addition, sirtuins and TOR have opposing effects on autophagy independently from each other. Thus sirutins and the downstream signalling pathway form a complex network. It will be important to study to address whether SIRT1 in the pituitary also controls the other endocrine axes and other downstream target involving aging and lifespan.

In thyrotropes, SIRT1 was found to be distributed in the cytosol, suggesting a role of SIRT1 in the cytoplasm (Fig. 5B). In fact, we confirmed the colocalization and binding between PIP5Kγ and SIRT1 in the pituitary gland (Fig. 5C). SIRT1 has been shown to shuttle between the cytoplasm and nucleus [54]. SIRT1 is localized in cytoplasm of pancreatic a cells and endothelial cells, and localized in nuclei in liver, muscle, and white adipose tissue [9,55]. Consistent with our findings that SIRT1 and PIP5Kγ were localized in the cytoplasm of pituitary gland cells, SIRT1 and endothelial nitric oxide synthase (eNOS) also colocalize and coprecipitate in cytoplasm of endothelial cells [55]. SIRT1 deacetylates eNOS for stimulating the activity and increases endothelial nitric oxide (NO) [55]. This result suggests SIRT1 has some targets in the cytoplasm as well as in the nucleus.

Our findings indicate that SIRT1 deacetylates acetylated PIP5Kγ at K265/K268, thereby activating TSH secretion from the pituitary gland (Fig. 7). The deacetylated form of PIP5Kγ is more enzymatically active and the PIP_2 synthesized the secretion

Figure 5. SIRT1 regulates TSH secretion via PIP5Kγ (K265/K268). (A) Western blot analysis of PIP5Kγ in the nerve tissues of mice. (B) Distribution of SIRT1 and PIP5Kγ in fractionated pituitary gland of mice. SIRT1 and PIP5Kγ were found to be present in cytosolic fractions rather than nuclear fractions. The first lane contains crude extract prior to separation. Nucleoporin, a nuclear marker; actin, a cytosolic fraction marker. (C) Double immunostaining with SIRT1 (red) and PIP5Kγ (green) in the anterior pituitary gland of rats. Scale bar, 10 mm. (D) Co-immunoprecipitation analysis of SIRT1 and PIP5Kγ in the cytosolic fraction of the pituitary gland of mice. Rabbit IgG was used as the negative control. (E) Western blot analysis of each PIP5K in PIP5Ks RNAi knockdown cells. (F) TLC analysis of [^{32}P]PIP_2 was extracted from PIP5Kγ RNAi pituitary cells metabolically labelled (for 24 h) with [^{32}P]orthophosphate. The PIP_2 level was corrected for the PC level ($n=5$). (G) Amounts of TSH secreted in each PIP5Ks RNAi knockdown cells ($n=16$). (H) Amounts of TSH secreted in cells overexpressing SIRT1 and PIP5Kγ RNAi ($n=16$). Empty vectors and random RNAi constructs were used as controls. Statistical analyses were performed using Fisher's PLSD test. *$P<0.05$ versus control. Values represent mean±SEM (% of control: F–H).
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of TSH from pituitary thyrotropes. K265 and K268 of PIP5Kγ correspond to R215 and R218 of PIP5Kβ in human, respectively. The region from Y209 to R215 in PIP5Kβ is a phosphatidic acid (PA)-binding site, and the kinase activity of PIP5Kβ is completely lost when basic residues in this site are substituted with acidic residues [56]. This loss of function is thought to be caused by the alteration of the electrostatic interactions in the pocket composed of the PA-binding site and the surrounding residues. Since acetylation of lysine residues causes the alteration of electrical charge, the acetylation-deacetylation cycle we describe can easily be understood to regulate the kinase activity of PIP5Kγ. Although our work points to SIRT1 as the deacetylase responsible for activating PIP5Kγ, we have no information of which protein acetyltransferase is involved in the inactivating (acetylation) reaction.

We also visualized the vesicle of EGFP-TSHβ on exocytosis in primary culture of pituitary cells by using TIRF microscopy. Because the TIRF microscope only visualizes vesicles just beneath the membrane, the traffic event is mainly reflecting exocytosis [27,57,58]. The number of disappeared TSH-containing granules within 5 s was increased in the SIRT1-overexpressing cells compared to the control cells (Fig. 2C and 2D), confirming the activation of vesicle trafficking. In accordance with our findings, previous work has shown that the absence of PIP5Kγ causes a reduction of the readily releasable vesicle pool and a delay of fusion pore expansion indicating a defect in vesicle priming in chromaffin cells [33]. Our assay system revealed that PIP5Kγ knockdown inhibited PIP_2 production and TSH secretion (Fig. 5G and 5H). Produced PI(4,5)P2 produced by PIP5Kγ is an important regulator of synaptic vesicle trafficking in nerve terminal [59]. Although SIRT1 has a many substrates, the SIRT1-PIP5Kγ pathway is important for intracellular trafficking and secretion in the thyrotropes. We also visualized the vesicle of EGFP-growth hormone (GH) on exocytosis in GH3 pituitary cells by using as TIRF microscopy (data not shown). Consistent with the observation of TSH-containing granules trafficking, the numbers of disappearing GH-containing granules was increased in the SIRT1-overexpressing cells (data not shown). These findings showed that the SIRT1-PIP5Kγ secretion pathway is shared by thyrotropes and other pituitary endocrine cells.

The physiological properties of the SIRT1 KO mouse are also consistent with the model proposed here. The levels of PIP5Kγ acetylation were elevated in SIRT1 KO mice and the TSH secretion levels were lower (Fig. 6D). SIRT1 KO mice have smaller pituitary than wild-type mice, however differentiation of the anterior pituitary occurs normally [39]. GH levels and insulin-like growth factor (IGF)-1 are not affected in SIRT1 KO mice [39]. In neuron-specific SIRT1 KO mice, the production and

Figure 6. High Ac-PIP5Kγ, low PIP_2, and low TSH secretion in pituitary glands obtained from SIRT1 KO mice. (A) Levels of acetylated PIP5Kγ in the pituitary of SIRT1 KO mice and WT mice. Endogenous PIP5Kγ was immunoprecipitated. The acetylated-lysine/PIP5Kγ level was measured ($n=3$). (B) TLC analysis of PIP_2 levels in the brain of SIRT1 KO mice and WT mice (WT: $n=6$, KO: $n=6$). (C) The number of TSHβ-immunopositive cells in SIRT1 KO mice and WT mice ($n=5$). (D) Pituitary concentrations of TSH in SIRT1 KO mice and WT mice ($n=10$). (E) Levels of TSH secreted by the pituitary cells in the organ culture over 24 h in SIRT1 KO mice and WT mice ($n=7$). (F) Plasma TSH levels per TSH-immnunopositive cell in SIRT1 KO mice and WT mice ($n=5$). (G) mRNA levels of anterior pituitary hormone in SIRT1 KO mice and WT mice. RNA levels were determined using RT-PCR analysis. GAPDH was used as a loading control ($n=3$). Values represent mean±SEM. Statistical analyses were performed using Student's t test or Mann-Whitney U test. *$P<0.05$ versus WT.
doi:10.1371/journal.pone.0011755.g006

secretion of GH but not other pituitary hormones is reduced [11]. The production of all pituitary hormones is regulated at least in part by signals from the hypothalamus. TSH-releasing hormone (TRH) secreted by the hypothalamus is the main stimulator of TSH synthesis and secretion [60,61]. It is generally considered that low serum TSH levels are attributable to the inhibition of TSH synthesis in the pituitary, which depletes the existing reserves of the hormone (exhausting the TSH "stock"), without changing the rate of secretion ("flow"). However, in this study, SIRT1 regulates release (related to "flow", not "stock") of TSH, suggesting that the physiological regulation of the pituitary-thyroid axis is dependent on regulated secretion.

The activity of SIRT1 is thought to be modulated by the nutritional status of the cell [62]. Recently, the expression of SIRT1 regulates oncogenic viral protein HPV7 [63] and locally acting IGF-1 isoform (mIGF-1), not circulating IGF-1 isoform [64]. Thus the activity and expression of SIRT1 changes topically and temporally. The core circadian rhythm regulator protein circadian locomotor output cycles kaput (CLOCK) is a histone acetyltransferase, and its activity is counterbalanced by NAD$^+$ and SIRT1 [65]. The circadian rhythm modulates metabolic activities on a long-term basis, while intracellular traffic regulation of TSH by SIRT1 might provide short-term control of metabolism dependent on nutrient availability.

Materials and Methods

Study subjects

SIRT1 KO mice were generated on a CD1 and 129/J mixed genetic background, and genotyped using a polymerase chain

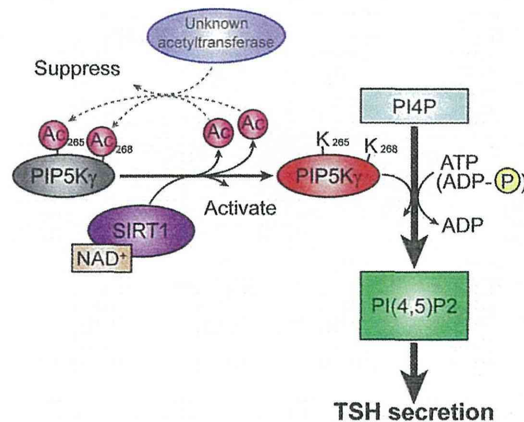

Figure 7. Model for the potential roles of SIRT1 in TSH secretion. Proposed model for regulation of TSH secretion by SIRT1.
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reaction (PCR)-based protocol [66]. Water and food was provided *ad libitum* for both groups. Human tissue sections were obtained from the Tokyo Medical and Dental University, and Tokyo Metropolitan Institute of Gerontology. These experiments were approved by the Mitsubishi Kagaku Institute of Life Science Ethics Committee. All experiments were reformed according to protocols approved by the Animal Care and Use Committee of Mitsubishi Kagaku Institute of Life Sciences.

Antibodies

We used the following antibodies: anti-SIRT1, β-actin, and Flag-M2 (Sigma-Aldrich); PIP5Kγ and nucleoporin (BD Transduction Laboratories); acetylated lysine (BioVision); GFP and agarose-conjugated GFP (Medical & Biological Laboratories); GAPDH (Millipore, Bedford, MA); ACTH (Nichirei), FSH and GH (DakoCytomation); TOTO-3 (Invitrogen). The anti-TSH, anti-LH, and anti-PRL antibodies were gifted by the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University. For immunoprecipitation, Kanaho's lab prepared polyclonal antibodies against mouse PIP5Kα, PIP5Kβ, and PIP5Kγ [37]. PIP4Kβ, prepared in Moses V. Chao's lab, was detected by using a polyclonal antibody [67]. Secondary antibodies were obtained from Jackson Immunoresearch and Molecular Probes.

Protein identification by LC-MS/MS analysis

Flag-SIRT1-associated complexes were digested with *Achromobacter* protease I, and the resulting peptides were analyzed using a nanoscale LC-MS/MS system. The criteria for match acceptance have been reported previously [31].

Plasmids and transfection

Plasmids encoding Flag-tagged SIRT1 were constructed using the pCMV-Tag2 or pCMV-Tag4 vectors (Stratagene). SIRT1 (H355A) was generated by mutating histidine 355 to alanine [29,30]. Plasmids encoding GFP-PIP5Ks or N-terminally epitope Flag-tagged PIP5Ks were constructed using the pcDNA3-GFP or pcDNA3-Flag (Invitrogen) vectors, as described previously [36]. The QuickChange Site-Directed Mutagenesis (Stratagene) kit was used to generate the PIP5Kγ (K265/268R) mutants. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. SIRT1 and PIP5K stealth RNAi were purchased from Invitrogen, and transfections were performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Rat TSHβ cDNA was generated by PCR. The PCR product was inserted into TagGFP (Evrogen). All constructs were confirmed by sequencing. The expression of TSHβ was confirmed by ELISA (Shibayagi), using lysates of GFP-TSHβ-overexpressing cells.

Immunoprecipitation

The transfected HEK293T cells (6-cm dish) were lysed in lysis buffer (25 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM ethylenediamine acetic acid (EDTA) and 0.2% Triton X-100) containing protease inhibitors, 0.5 μM trichostatin A, and 5 mM NAM. After brief sonication, the lysates were cleared by centrifugation. The immunoprecipitates thus obtained were analyzed by immunoblotting. Cytoplasmic proteins obtained from rat pituitary were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instructions (Pierce).

Western blot analysis

Lysate proteins were separated on an 8% SDS-PAGE gel and then blotted onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked using Block Ace (Dainippon Seiyaku) and probed with the appropriate primary antibodies. The bound primary antibodies were detected using the corresponding horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using the ECL kit (GE Healthcare Bio-

Sciences). The signals were quantified using the ImageJ imaging software.

Primary pituitary culture, electroporation and hormone assay

Pituitary cell cultures were prepared from the anterior pituitary glands of 12- to 15-week-old male Wistar-Imamichi rats. The pituitary glands were finely minced and then digested by rapid agitation in DMEM containing 4 mg/mL collagenase (type 2; Worthington), 2 mM L-glutamine, 25 mM HEPES, and 400 mg/mL DNase for 30 min at 37°C [68]. The pituitary cells were transfected using a microporator (Digital Bio Technology). In all, 1.5×10^5 cells were electroporated with DNA (0.5 μg) or RNA (100 nM) at 1,500 V and a pulse width of 30 ms and seeded in 24-well plates (hormone assay) or 35-mm dishes (TIRF analysis). The cells were incubated in high-glucose DMEM containing 5% FBS and 15% horse serum at 37°C under 5% CO_2. At 2 days after electroporation, the pituitary cells were preincubated in a buffer containing low amounts of K^+ (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES [pH 7.4] and 11 mM glucose) for 30 min, and then incubated in the same buffer for 24 h at 37°C. The levels of the hormones released into the culture medium were measured using radioimmunoassay kits for rat TSH (Shibayagi). The cells were harvested using celLytic buffer M (Sigma-Aldrich) containing protease inhibitors (Complete EDTA free; Roche Diagnostics). After removal of the cellular debris, the protein concentrations in the lysates were determined using the Bradford method. The pituitary cells were observed by TIRF confocal microscopy 1 day after electroporation. The plasma concentrations of mouse TSH were measured using radioimmunoassay kits (National Institute of Diabetes and Digestive and Kidney Diseases).

TIRF microscopy

For TIRF microscopy, a total internal reflection system (Nikon) was used with minor modifications. Light from an Ar laser (488 nm) was introduced into an inverted epifluorescence microscope through a single-mode fiber and a double-illumination lens. The cells were observed in the thermostat-controlled stage (37°C).

PIP5K acetylation assay and *in Vitro* deacetylation assay

HEK293T cells were transfected with Flag-PIP5Kγ, Flag-PIP5Kγ (K265/268R), Flag-PIP5Kα, or Flag-PIP5Kβ and treated with 0 mM or 5 mM NAM (Sigma-Aldrich). After 24 h, the cells were placed in celLytic buffer M and immunoprecipitated. The Flag-PIP5Ks were eluted using the Flag peptide. The eluted Flag-PIP5Ks were combined with either recombinant SIRT1 or SIRT1 (H355A) in a reaction buffer (50 mM Tris-HCl [pH 8.8], 4 mM $MgCl_2$ and 0.5 mM DTT), 100 μM of NAD^+ was added, and the complexes were incubated for 3 h at 30°C as described [30]. The reaction mixture was used for measuring the PIP5Kγ activity.

Measurement of PIP5Kγ activity

The PIP5Kγ activity was determined according to the procedure described in a previously reported [36]. Briefly, 0.1 pmol of PIP5Kγ was incubated at 37°C for 25 min in 50 mL of 50 mM Tris-HCl, (pH 7.5), 1 mM EGTA, 10 mM $MgCl_2$ and 0.004% (w/v) NP-40 containing 50 μM PI(4)P and 50 mM [γ-^{32}P]ATP (0.1 μCi/assay). After extraction using the Bligh and Dyer method, the lipids were separated by thin-layer chromatography (TLC) as previously reported [36]. The [^{32}P]PI(4,5)P_2 produced was analyzed with an FLA2000 Bio-imaging analyzer (Fuji Photo Film).

Lipid biochemistry

The HEK293T cells or pituitary cells were spread on a 6-cm dish at concentrations of 5×10^5 cells/well and the plates were incubated for 24 h. These cells were transfected or electroporated with SIRT1 stealth RNAi incubated with 4mCi of [^{32}P]orthophosphate in phosphate-free DMEM. After 48 h of incubation, the lipids were extracted using chloroform:methanol:1N HCl (3:3:1, v/v). The lipids were dissolved in chloroform:methanol (2:1) before being spotted onto a TLC plate (silica gel 60; Merck). The plate had been sprayed with potassium oxalate, using a solvent system comprising 1.0% potassium oxalate in methanol:water (2:3, v/v), and pre-activated by heating at 110°C for 60 min before the spotting. The chromatogram was developed in chloroform:methanol:20% methylamine (60:36:10, v/v/v). The radioactivity was visualized using a Fuji FLA2000 bioimaging analyzer. Lipids were extracted from the mouse brain, corrected for tissue weight, and TLC was performed as described above. Each spot was visualized using 0.005% primuline, a fluorescent dye. The signals were quantified using ImageJ imaging software.

Immunohistochemistry

The isolated mouse and rat organs and the cultured cells were fixed with 4% paraformaldehyde and processed for immunostaining as described previously [69,70]. Human pituitary glands were obtained by concluding autopsies on the bodies of 20 individuals aged 20—103 years at the time of death. None of these individuals had a clinical history of abnormal endocrine dysfunction. Half of each pituitary gland was serially sectioned and the sections were scanned at a magnification of 40*X*. The total number of cells and the number of TSH-immunopositive cells in the entire area in each field-of-view were counted [71].

RT-PCR

Total RNA was extracted using Sepasol reagent (Nakalai Tesque), precipitated by ethanol in the presence of ethachinmate (Nippon Gene), and reverse transcribed using ReverTraAce (TOYOBO). We used the following primers: 5′-CGTCCCGTAGACAAAATGGT-3′ and 5′-GAATTTGCCGTGAGTGGAGT-3′ for mouse GAPDH, 5′-CGCATACGAGTGGAGAGAAA-3′ and 5′-ATGGCGACAGGGAAGGAGAA-3′ for mouse TSHb, 5′-CAGCCTGATGTTTGGTACCTCGGA-3′. Signals were quantified using ImageJ software.

Statistical analysis

All the results are expressed as mean±SEM. The statistical significance of the differences between the groups was examined using Student's *t*-test, Mann-Whitney *U* test, or repeated-measures analysis of variance (ANOVA), as appropriate, and the STATVIEW program. When a significant F ratio was obtained, we conducted post-hoc analysis by using Fisher's protected least-significant difference (PLSD) test. $P<0.05$ was considered to be significant.

Supporting Information

Video S1 Real-time motion of EGFP-TSHβ granules close to the plasma membrane of the pituitary cells was monitored at 5 s intervals by TIRF microscopy. Scale bar, 20 μm.
Found at: doi:10.1371/journal.pone.0011755.s001 (0.06 MB AVI)

Video S2 Real-time motion of EGFP-TSHβ granules close to the plasma membrane of the SIRT1-overexpressing pituitary cells was monitored at 5 s intervals by TIRF microscopy. Scale bar, 20 μm.

Found at: doi:10.1371/journal.pone.0011755.s002 (0.06 MB AVI)

Video S3 Real-time motion of EGFP-TSHβ granules close to the plasma membrane of SIRT1-RNAi pituitary cells was monitored at 5 s intervals by TIRF microscopy. Scale bar, 20 μm.
Found at: doi:10.1371/journal.pone.0011755.s003 (0.06 MB AVI)

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Author Contributions

Conceived and designed the experiments: SAA LG MS. Performed the experiments: SAA NZ TK IY TH SiI YE MK SYS. Analyzed the data: SAA NZ KI TK IY TH SiI HY TN. Contributed reagents/materials/analysis tools: RS TY KI TC IS AM AM MS MVC YK MM. Wrote the paper: SAA NZ KI TK IY TH SiI RS TY YE MK KI TC HY IS SYS AM AM MS MVC MT YK TN HS YD MM LG MS.

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Germline alterations in the *CDH1* gene in familial gastric cancer in the Japanese population

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Germline point or small frameshift mutations of the *CDH1* (*E-cadherin*) gene are known to cause familial gastric cancer (FGC), but the frequency of *CDH1* mutations is low in Japanese patients with FGC. Because recent studies have reported germline large genomic deletions of *CDH1* in European and Canadian patients with FGC, in the present study we examined DNA samples from 13 Japanese patients with FGC to determine whether similar germline changes were present in *CDH1* in this population. Using a sequencing analysis, a 1-bp deletion (c.1212delC), leading to the production of a truncated protein (p.Asn405IlefsX12), was found in an FGC family; immunohistochemical analysis revealed the loss of CDH1 protein expression in the tumors in this family. Using a combination of multiplex ligation-dependent probe amplification (MLPA) and RT-PCR analyses, we also found a large genomic deletion (c.164-?_387+?del), leading to the loss of exon 3 and the production of a truncated protein (p.Val55GlyfsX38), in another FGC family. The functional effects of the detected mutations were examined using a slow aggregation assay. Significant impairment of cell–cell adhesion was detected in CHO-K1 cells expressing Ile405fsX12- and Gly55fsX38-type CDH1 compared with cells expressing wild-type CDH1. Our results suggest that the p.Asn405IlefsX12 and p.Val55GlyfsX38 mutations of the *CDH1* gene contribute to carcinogenesis in patients with FGC. This is the first report of *CDH1* germline truncating mutations in Japanese patients with FGC. Screening for large germline rearrangements should be included in *CDH1* genetic testing for FGC. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.02038.x, 2011)

Gastric cancer is one of the most common cancers worldwide and is divided histopathologically into two types: intestinal (differentiated) and diffuse (undifferentiated).[1] Hereditary diffuse gastric cancer (HDGC) is a cancer predisposition syndrome dominated by diffuse gastric cancer, and the *CDH1* (*E-cadherin*) gene is known to be responsible.[2,3] The *CDH1* gene is a calcium-dependent cell adhesion molecule and is one of the most important tumor suppressor genes in gastric cancer.[4] Sequencing analyses have revealed germline *CDH1* mutations in approximately 30% of patients with HDGC, as determined using the criteria for HDGC defined by the International Gastric Cancer Linkage Consortium (IGCLC).[5,6] However, the detection rate of *CDH1* germline mutations in Japanese patients with familial gastric cancer (FGC) is low compared with that in European patients.[7–10] The difference in the detection rate may be due to differences in the contribution of environmental factors, because the incidence of gastric cancer is relatively high in Japan.[1,5] As another possibility, gross *CDH1* genomic rearrangements that cannot be detected using conventional sequencing analysis may be responsible for FGC in Japanese patients. Large genomic rearrangements have recently been reported to cause susceptibility to several hereditary cancers, such as those of the *MLH1* or *MSH2* genes in patients with Lynch syndrome,[11] the *APC* gene in patients with familial adenomatous polyposis,[12] and the *BRCA1* gene in patients with familial breast cancer.[13] Based on these findings, we hypothesized that large genomic rearrangements of the *CDH1* gene may be responsible for a subset of FGC in the Japanese population. In agreement with this hypothesis, Oliveira *et al.*[14] recently collected European and Canadian FGC patients in which no germline *CDH1* mutations were detected using a sequencing analysis and identified some FGC families with germline large genomic deletions of the *CDH1* gene. Therefore, in the present study, we examined 13 Japanese FGC families for the possible presence of large genomic rearrangements, as well as germline point or small frameshift mutations, in the *CDH1* gene. We identified a germline 1-bp deletion (c.1212delC) and a germline large genomic deletion (c.164-?_387+?del), both of which led to the production of a truncated CDH1 protein, and functionally characterized these CDH1 mutant proteins.

Materials and Methods

Tissue samples, cell line, and nucleic acid extraction. Blood samples and paraffin-embedded tissues were collected from 13 FGC families fulfilling the revised clinical criteria for HDGC.[5] The Chinese hamster ovary (CHO)-K1 cell line was purchased from Human Science Research Resource Bank (Osaka, Japan). Cells were cultured in α-minimum essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS under a 5% CO_2 atmosphere at 37°C. Genomic DNA was extracted using a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA) or a DNeasy Tissue Kit (Qiagen). Total RNA was extracted with a PAXgene Blood RNA Kit (Qiagen) or with an RNeasy Plus Mini Kit (Qiagen). All samples were analyzed in a blinded manner. The research protocol was approved by the institutional review boards of Hamamatsu University School of Medicine and the relevant institutes.

Polymerase chain reaction and sequencing analysis. Fragments covering all coding exons and boundary regions of the *CDH1* gene were amplified using PCR. Platinum Taq PCRx DNA polymerase (Invitrogen, Carlsbad, CA, USA) was used for the amplification of exon 1, whereas HotStarTaq DNA polymerase (Qiagen) was used for the amplification of exons 2–16. Information on the primer sequences and PCR conditions have been described previously.[7,10,15] The PCR products were

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purified with Exo-SAP-IT (GE Healthcare Bio-Science, Piscataway, NJ, USA) and sequenced directly using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and the ABI 3100 Genetic Analyzer (Applied Biosystems).

Immunohistochemical analysis. Immunohistochemical analysis was performed as described previously.[16] Briefly, paraffin block sections derived from patients who underwent a gastrectomy and/or autopsy were immunostained with a monoclonal antibody (mAb) against CDH1 (clone 36B5; epitope, N-terminal amino acid sequence; Novocastra, Newcastle, UK). Sections were also stained with H&E.

Multiplex ligation-dependent probe amplification analysis. Twelve FGC patients negative for germline point or small frameshift mutations in the *CDH1* gene were tested for large genomic deletions in the *CDH1* gene using the SALSA P083-B1 *CDH1* multiplex ligation-dependent probe amplification (MLPA) kit (MRC-Holland, Amsterdam, The Netherlands). The reactions were performed according to the manufacturer's instruction. Probe ratios below 0.7 and above 1.3 were regarded as indicative of a decrease and increase, respectively, in the gene dosage.

Reverse transcription–polymerase chain reaction. Total RNA was converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The following sets of primers were used in the PCR amplification: 5′-AGG TCT CCT CTT GGC TCT GC-3′ and 5′-CAG CTG ATG GGA GGA ATA ACC-3′ for the CDH1 transcripts; and 5′-TGG GCC AGA AGG ACT CCT AC-3′ and 5′-GCA TGA GGG AGA GCG TAG C-3′ for the β-actin transcripts. The PCR products were fractionated using electrophoresis on a 2.0% agarose gel and stained with ethidium bromide; the gel was then examined under UV light. A 100-bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used. Any PCR products exhibiting multiple bands were sequenced after subcloning with a pGEM-T Easy vector system (Promega, Madison, WI, USA).

Plasmid construction. Wild-type and exon 3 deletion-type *CDH1* cDNA were inserted into a pIRESpuro2 mammalian expression vector (Clontech, Palo Alto, CA, USA). The expression vectors for the Ile405fsX12-type and Leu415-type CDH1 were generated using site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The sequences of all vectors were confirmed by sequencing.

Establishment of CHO-K1 cell lines stably expressing human CDH1. The wild-type or mutant-type CDH1 plasmid vector was transfected into CDH1-negative CHO-K1 cells[17] using Lipofectamine 2000 reagent (Invitrogen). Puromycin-resistant clones were isolated by culturing in medium containing 5 μg/mL puromycin (Clontech). Positive clones were confirmed using a combination of RT-PCR, western blot, and immunofluorescence analyses.

Western blot analysis. Cells were harvested in lysis buffer containing 10 mM HEPES (pH 7.5), 1.0% Nonidet P-40, 1 mM EDTA, 1 mM DTT, and 0.1 mg/mL PMSF. The whole-cell extracts were mixed with an equal volume of 2× SDS sample buffer and boiled. A 25-μg aliquot of the extract was subjected to SDS-PAGE and the proteins obtained were transferred electrophoretically to a PVDF membrane (GE Healthcare Bio-Science). Membranes were blocked with non-fat milk and incubated with an anti-CDH1 mAb (clone SHE78–7; epitope, the first extracellular domain;[18] Takara Bio, Shiga, Japan) or anti-β-actin mAb (Abcam, Cambridge, UK). After washing with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), membranes were incubated with anti-mouse HRP-conjugated secondary antibody (GE Healthcare Bio-Science). After washing with TBS-T, immunoreactivity was visualized with an ECL chemiluminescence system (GE Healthcare Bio-Science).

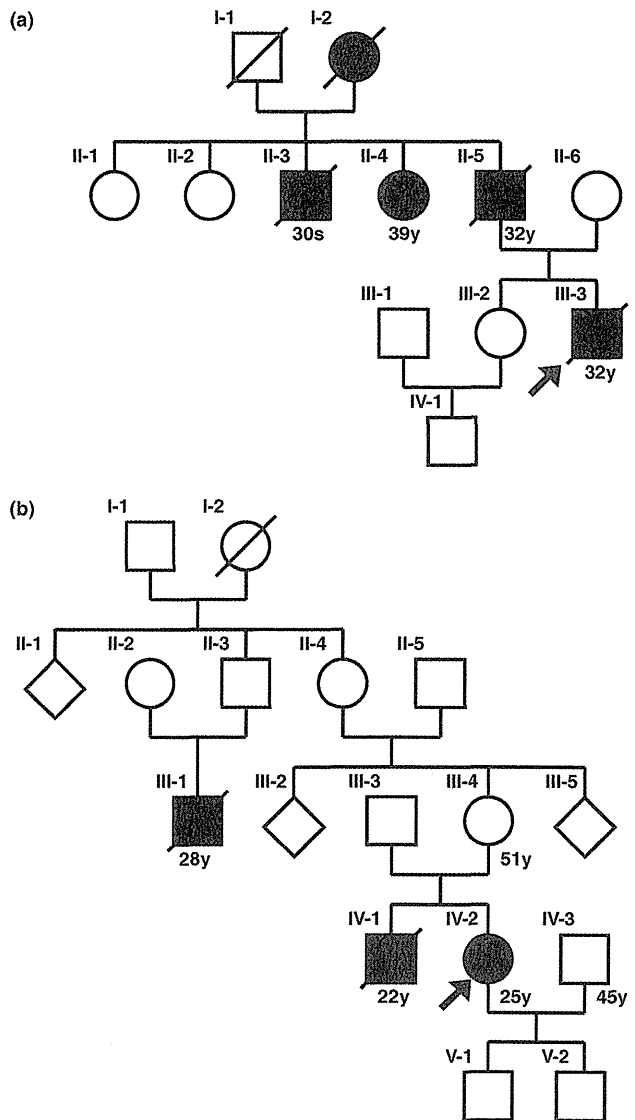

Fig. 1. Pedigrees of familial gastric cancer (FGC) families with germline *CDH1* alterations, specifically (a) a c.1212delC (p.Asn405IlefsX12) germline mutation of the *CDH1* gene and (b) a c.164-?_387+?del (p.Val55GlyfsX38) germline mutation of the *CDH1* gene. Squares indicate males; circles indicate females. Solid symbols indicate gastric cancer patients. Symbols with a slash indicate deceased individuals. Arrows point to the probands. The numbers below the symbols indicate the age at diagnosis for affected family members or the age at the time of analysis for unaffected family members.

Indirect immunofluorescence analysis. The CHO-K1 clones were fixed, permeabilized, and blocked with goat serum. Cells were incubated with anti-CDH1 mAb SHE78–7 (epitope, the first extracellular domain;[18] Takara Bio) at room temperature for 1 h, and indirect immunofluorescence labeling was performed at room temperature for 1 h with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO, USA). Immunostained cells were examined, digitized, and stored as described previously.[19]

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Slow aggregation assay. Slow aggregation assays were performed as described previously.[20] Cells were trypsinized and transferred to an agar gel in a 96-well plate. After 48 h, aggregate formation was evaluated using an inverted microscope. The entire area was divided into ''units'' using a square mesh, with each unit containing a maximum of 30–40 cells. Quantification of the aggregate was estimated using the following formula:

$$\text{Aggregate (\%)} = \text{(no. units with cells occupying} \geq 50\% \text{ of the unit)/(total no. units)} \times 100$$

Statistical analysis. Statistical analyses were performed using Dunnett's multiple comparison test with JMP version 7.01 software (SAS Institute, Cary, NC, USA).

Results

Identification of two germline mutations of the *CDH1* gene in Japanese FGC patients. Of the 13 FGC families evaluated in the present study, we had shown previously that six were negative for germline point or small frameshift mutations in the *CDH1* gene;[10,15] therefore, in the present study, we screened the probands of seven FGC families for germline *CDH1* mutations using PCR and subsequent sequencing analysis. Representative pedigrees are shown in Figure 1. One heterozygous c.1212delC mutation at the *CDH1* gene locus was found in one male proband (III-3) who was affected with signet-ring cell carcinoma of the stomach at 32 years of age (Figs 1a,2a). A deletion of one nucleotide (c.1212C) in exon 9 resulted in a frameshift at codon 404, the introduction of 11 novel amino acids, and the premature termination of a 415-amino acid protein (p.Asn405Ile-fsX12). A gastric cancer had also been recorded in four other family members (I-2, II-3, II-4, and II-5), and the c.1212delC mutation was detected in III-3's aunt (II-4), who had gastric cancer, using sequencing analysis (Fig. 2a). The c.1212delC mutation had been found previously in one northern European FGC family,[5] meaning that this is the first case of the germline mutation in Asia. Immunohistochemical analysis showed the loss of CDH1 protein expression in cancerous gastric tissue from the proband and his aunt, indicating that a second hit had occurred in the remaining wild-type *CDH1* allele (Fig. 2b).

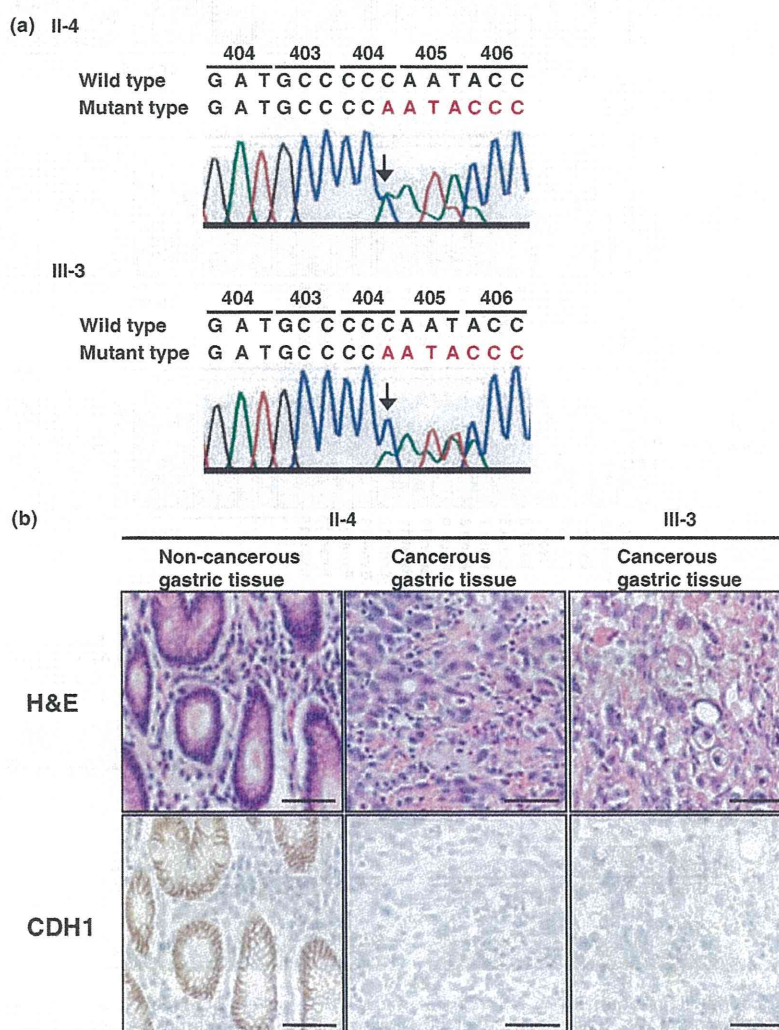

Fig. 2. Identification of the c.1212delC (p.Asn405IlefsX12) germline mutation of the *CDH1* gene. (a) Detection of the germline *CDH1* mutation in DNA from the male proband (III-3) and his aunt (II-4) using direct sequencing analysis. Both electropherograms of *CDH1* exon 9 show a heterozygous 1-bp deletion (c.1212delC). An arrow points to the position of the 1-bp deletion at codon 404. (b) Immunohistochemical analysis of the CDH1 protein in gastric cancers from this family. Non-cancerous tissue and cancerous gastric tissue samples from patient II-4 and cancerous gastric tissue from patient III-3 are shown. Scale bars, 50 μm.

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Next, we screened the remaining 12 FGC patients who were negative for germline point or small frameshift mutations of *CDH1* for large genomic rearrangements in the *CDH1* gene using MLPA analysis. A decreased signal at *CDH1* exon 3, suggesting a heterozygous deletion of a portion of *CDH1*, was detected in one female proband (IV-2) affected with signet-ring cell carcinoma of the stomach at 25 years of age (Figs 1b,3a). The decreased signal was also detected in a blood sample from the proband's mother (III-4) and a paraffin-embedded duodenal sample from the proband (Fig. 3a). Among the proband's family members, her brother (IV-1) and a male cousin of her mother (III-1) were diagnosed with gastric cancer at 22 and 28 years of age, respectively. To determine the effect of a reduced signal for exon 3, as detected using MLPA analysis, on the *CDH1* mRNA transcript, RT-PCR was performed with a set of primers for the sequences on CDH1 exons 2 and 4. An aberrant band was detected in cDNA from the proband, and subsequent sequencing analysis confirmed that exon 3 of the CDH1 transcript was heterozygously deleted in this case (Fig. 3b–d); thus, a heterozygous c.164-?_387+?del mutation existed in the proband. The c.164-?_387+?del mutation was predicted to cause the production of a truncated protein (p.Val55GlyfsX38). As far as we know, the c.164-?_387+?del mutation observed in this case has not been reported previously as a germline mutation, indicating that it is a novel *CDH1* germline mutation.

Establishment of various stable CDH1 transfectants. To better understand the relationship between the *CDH1* mutations detected and the familial clustering of gastric cancer, we

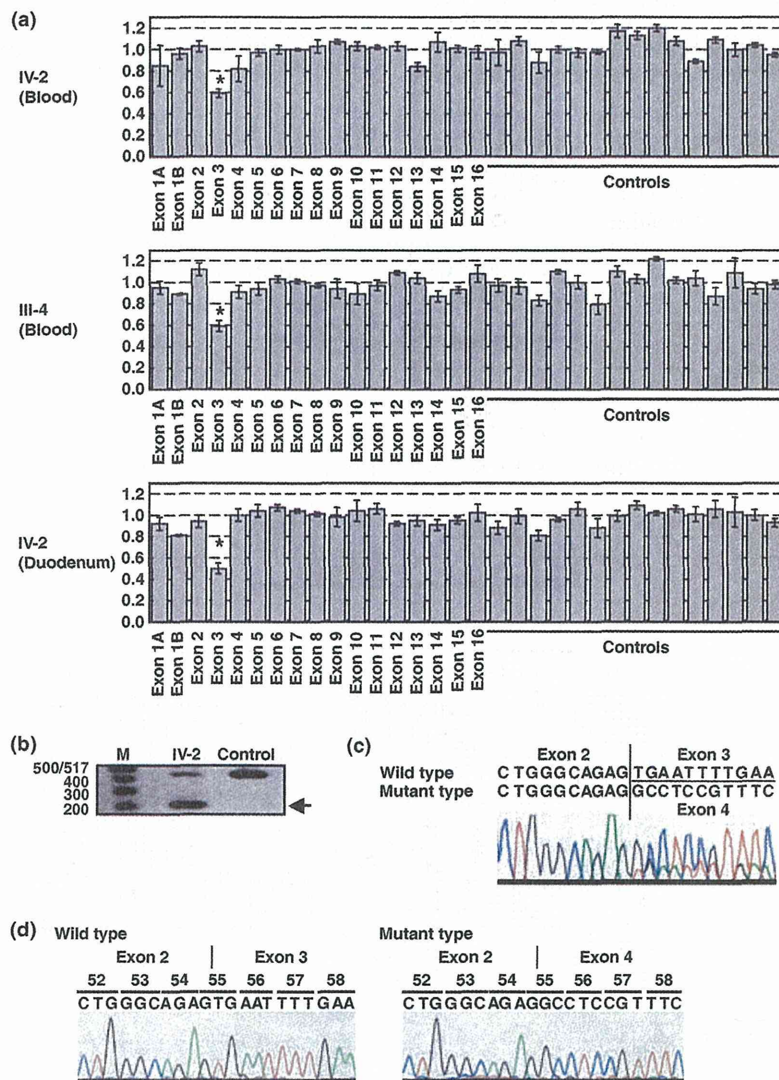

Fig. 3. Identification of large genomic deletion (c.164-?_387+?del) of the *CDH1* gene. (a) Detection of a decreased signal (asterisk) at *CDH1* exon 3 in DNA from the female proband (IV-2) and her mother (III-4) using multiplex ligation-dependent probe amplification (MLPA) analysis. The names of the MLPA probes are shown below the panels. Data are shown as the mean ± SD. (b–d) Identification of *CDH1* exon 3 deletion using RT-PCR and sequencing analyses. (b) A blood sample from patient IV-2 was subjected to RT-PCR with a set of primers for a sequence spanning exons 2 and 4 of *CDH1*; the products were subsequently electrophoresed on an agarose gel. The arrow indicates a band that is smaller than the band corresponding to the calculated size of the wild-type sequence. An individual not showing an abnormal signal in the MLPA analysis for *CDH1* was used as a control. M, DNA size marker. (c) Direct sequencing analysis of the RT-PCR product from Patient IV-2. (d) Sequencing analysis of the subcloned RT-PCR product from patient IV-2.

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attempted to characterize the mutant CDH1 proteins (Gly55fsX38-type and Ile405fsX12-type) functionally. The Leu415-type CDH1 was also included in this analysis, because: (i) a germline mutation causing the production of the Leu415-type mutant CDH1 protein was reported previously in Japanese patients with FGC;[5] (ii) this amino acid substitution is predicted to affect protein function according to the Sorting Intolerant From Tolerant (SIFT) program;[21] and (iii) it has never been characterized *in vitro*. We transfected a human CDH1 expression vector into CHO-K1 cells lacking CDH1 expression[17] and CDH1 stable transfectants were isolated using puromycin selection (Fig. 4). The expression of the mRNA transcript and the protein of ectopic CDH1 was confirmed using a combination of RT-PCR, western blot, and immunofluorescence analyses (Fig. 4b–d). Regarding intracellular localization, membranous expression was detected in cells expressing the wild-type or Leu415-type CDH1, but not in cells expressing the Ile405fsX12-type or Gly55fsX38-type CDH1 (Fig. 4d). Through all the above analyses, five different CHO-K1 clones, including an empty vector-transfected clone, were established successfully.

Functional characterization of mutant CDH1 proteins. Because cell–cell adhesion is a major function of the CDH1 protein, we compared levels of homotypic cell adhesion between the five CHO-K1 clones using a slow aggregation assay. Clones expressing the Gly55fsX38-type or Ile405fsX12-type CDH1 and an empty vector-transfected clone produced significantly fewer aggregations than the clone expressing wild-type CDH1 (Fig. 5). The quantity of cell–cell aggregation did not differ significantly between clones expressing wild-type and Leu415-type CDH1 (Fig. 5). These results suggest that Gly55fsX38-type and Ile405fsX12-type CDH1, but not Leu415-type CDH1, have an impaired cell–cell adhesion function.

Discussion

In the present study, DNA samples from 13 Japanese FGC families were examined for germline alterations in the *CDH1* gene. Using sequencing analysis, a 1-bp deletion (c.1212delC: p.Asn405IlefsX12) was detected in an FGC family; in another FGC family, a large genomic deletion (c.164-?_387+?del: p.Val55GlyfsX38) was detected using MLPA and RT-PCR

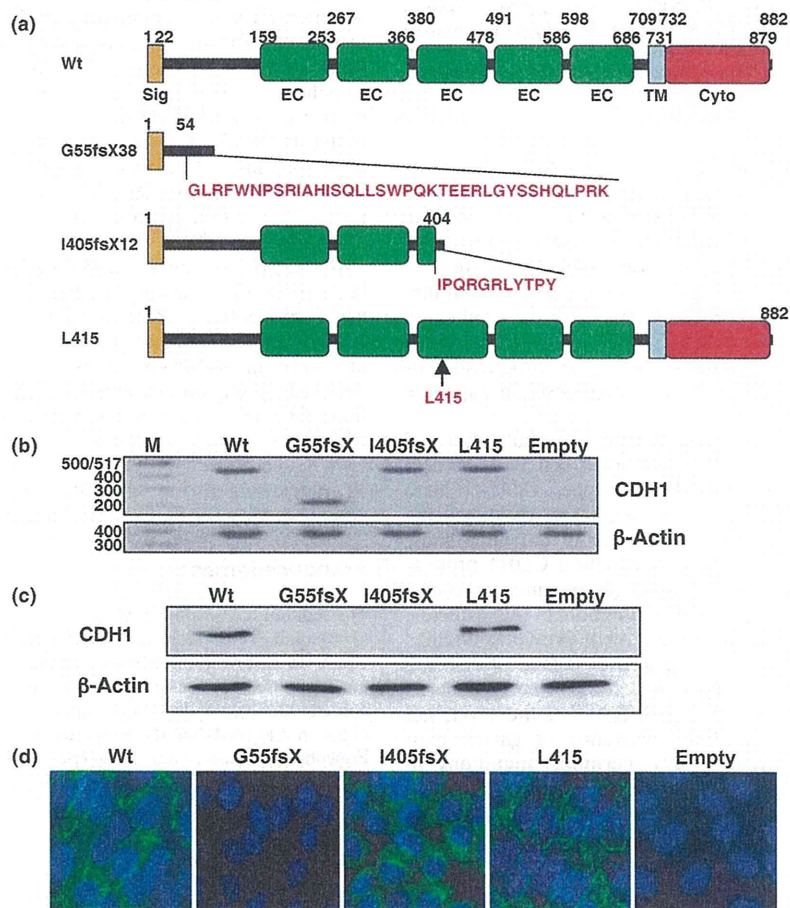

Fig. 4. Establishment of CHO-K1 cell lines stably expressing human CDH1 protein. (a) Scheme of the wild-type (Wt) and mutant (G55fsX38, I405fsX12, and L415) human CDH1 proteins. Amino acid sequences newly created by CDH1 genetic alterations are denoted in red in each mutant. Sig, signal peptide; EC, extracellular domain; TM, transmembrane domain; Cyto, cytoplasmic domain. (b) Detection of the expression of wild-type and mutant human *CDH1* mRNA transcripts in stable CHO-K1 clones using RT-PCR analysis, which was performed for each clone isolated using a set of primers for a sequence spanning exons 2 and 4 of *CDH1* to confirm ectopically expressed CDH1. Empty, empty vector-transfected clone. In addition, mRNA transcripts of the Chinese hamster β-actin were amplified as an internal control. M, DNA size marker. (c,d) Detection of the expression of wild-type and mutant human CDH1 protein in stable CHO-K1 clones using western blot analysis (c) and immunofluorescence analysis (d). Chinese hamster β-actin protein was used as an internal control. In the immunofluorescence analysis, CDH1 protein (green) was immunostained with an anti-CDH1 primary antibody and an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (blue).

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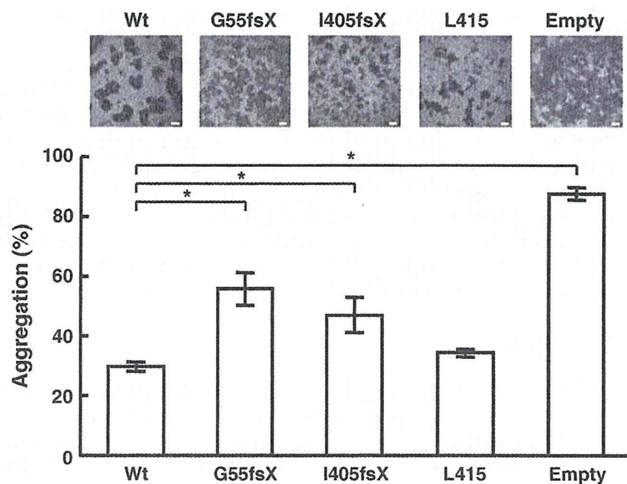

Fig. 5. Evaluation of the cell adhesion function of mutant-type CDH1 using a slow aggregation assay. Representative photographs of the results of a slow aggregation assay of CHO-K1 clones stably expressing wild-type (Wt) or mutant (G55fsX38, I405fsX12, and L415) human CDH1 are shown above the graph. Empty, empty vector-transfected clone. Data are the mean ± SD of four independent experiments. $*P < 0.05$. Scale bars, 100 μm.

analyses. Both germline changes led to the production of a truncated CDH1 protein. When the level of cell–cell adhesion was compared between wild-type CDH1 and these two mutant CDH1 proteins using a slow aggregation assay, significantly impaired cell–cell adhesion function was detected for the two mutant proteins. These results suggest that the c.1212delC mutation in the *CDH1* gene, which is associated with the production of p.Asn405IlefsX12, and the c.164-?_387+?del mutation in the *CDH1* gene, which is associated with the production of p.Val55GlyfsX38, are involved in carcinogenesis in Japanese patients with FGC.

Using a slow aggregation assay, the present study detected a defect in the cell–cell adhesion function in cell lines harboring Gly55fsX38-type and Ile405fsX12-type CDH1. Both mutants are truncated CDH1 proteins; therefore, they lack the entire or part of the extracellular domain, transmembrane domain, and cytoplasmic domain of the mature CDH1 protein. The absence of these domains in both types of mutant proteins is considered responsible for their functional impairments. Because these results are in agreement with previous findings that germline *CDH1* changes leading to a truncated protein are present in FGC patients of various ethnicities,[3] we considered that the p.Val55GlyfsX38 and p.Asn405IlefsX12 mutations are pathogenetic towards the familial occurrence of gastric cancers. All previously reported *CDH1* germline mutations in Japan are missense types (p.Gly62Val, p.Ile415Leu, and p.Val832Met);[7,9,10] therefore, the present report is the first description of a *CDH1* germline mutation associated with the production of a truncated CDH1 protein in Japanese patients with FGC.

In the recent review by Cisco *et al.*,[22] it was reported that the penetrance of diffuse gastric cancer in patients who carry a *CDH1* mutation is estimated to be 63–83% for women and 40–67% for men. In the family with the c.164-?_387+?del *CDH1* mutation (Fig. 1b), III-1, IV-1, and IV-2 were affected with gastric cancer in their 20s, however, II-3, II-4, and III-4 were unaffected, implying the penetrance of gastric cancer in this family seems slightly lower than the penetrance described by Cisco *et al.*[22] However, because some information, such as endoscopic surveillance data for II-3, II-4, and III-4, are unavailable and it is possible that new cases of gastric cancer may develop in the family members in future, it is important to investigate this kind of family more precisely and for a longer period of time to determine the correct penetrance.

In the present study, *CDH1* germline alterations were detected in two of 13 Japanese FGC families (15.4%) who fulfilled the revised clinical criteria for HDGC according to the IGCLC. What is responsible for the gastric cancer in the remaining FGC families? One possible explanation is the contribution of environmental factors, such as infection with *Helicobacter pylori* (*H. pylori*) and the intake of salted/smoked and pickled/preserved foods (rich in salt, nitrites, and preformed nitroso compounds),[23] to the development of FGC. In fact, the incidence of *H. pylori* infection is known to be high in Japan.[24] Another possible explanation is the existence of other genes responsible for the development of FGC. So far, germline mutations of the *p53*, *MET*, and *STK11* genes and genes causing Lynch syndrome have been detected in the familial clustering of gastric cancer.[25–29] However, the incidence of these germline mutations is likely to be lower than that of the *CDH1* germline mutation, suggesting that previously unidentified genes responsible for FGC may exist. Future investigation using the ''omics'' approach or whole genome sequencing may identify novel genes responsible for FGC.

In the present study, MLPA analysis was performed to detect large genomic rearrangements, which are difficult to detect using conventional PCR sequencing, at the *CDH1* gene locus. Indeed, an abnormal signal was detected in MLPA analysis in one case in which no germline *CDH1* mutations had been detected by sequencing analysis. Therefore, similar to the detection of genomic rearrangements in the *MLH1* and *MSH2* genes, which has been proposed to be included in genetic testing for Lynch syndrome,[30] we propose that screening for germline large rearrangements of *CDH1* should be included in *CDH1* genetic testing for FGC in the Japanese population.

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Disclosure statement

The authors have no conflicts of interest.

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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).

Lung cancer susceptibility

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

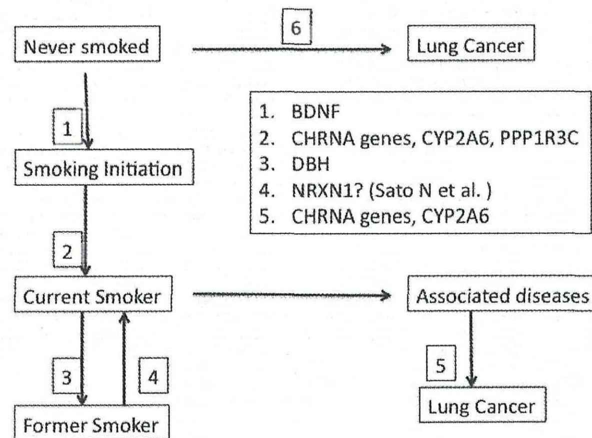

Figure 1. Presumed steps and genetic influences in the path from smoking to lung cancer. (Modified from (101). Amos CI, Spitz MR, Cinciripini P: Chipping away at the genetics of smoking behavior.) 1. Smoking initiation, 2. Smoking dosage, 3. Smoking cessation, 4. Difficulty quitting smoking, 5. Smoking 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.

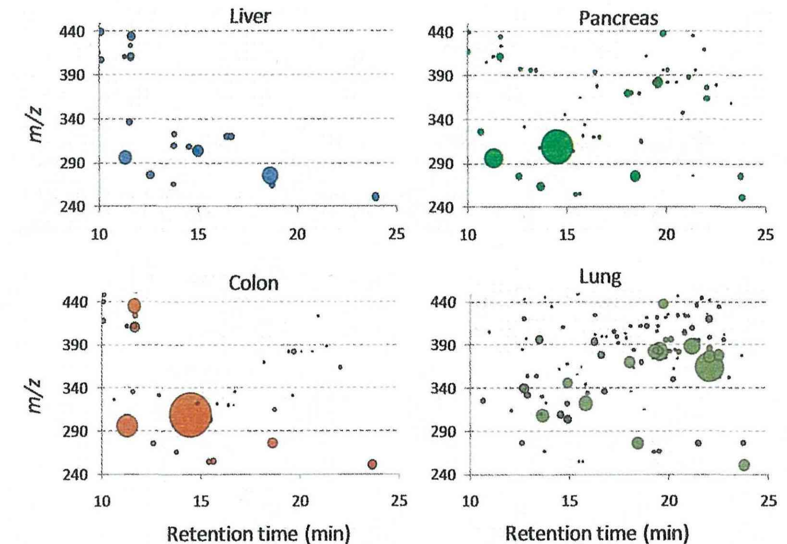

Figure 2. Adductome maps of 4 organs of the same individual. The position of each circle represent the species of the DNA adducts, and the size of each circle indicates the relative quantity of each DNA adduct. The adductome map shows that each organ contains more than one type of DNA adduct. The profile 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.

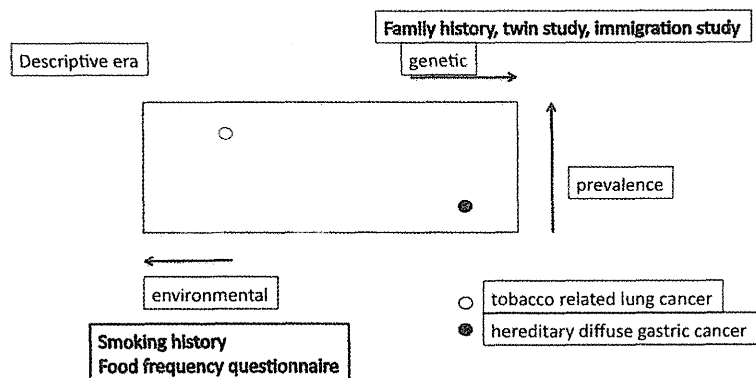

Figure 3. Methodological developments in nature and nurture analyses of cancer susceptibility. The arrows how much environmental and genetic components influence the incidence of diseases. A disease like lung cancer (open circle) would be located in a more environmental position in the spectrum, whereas 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

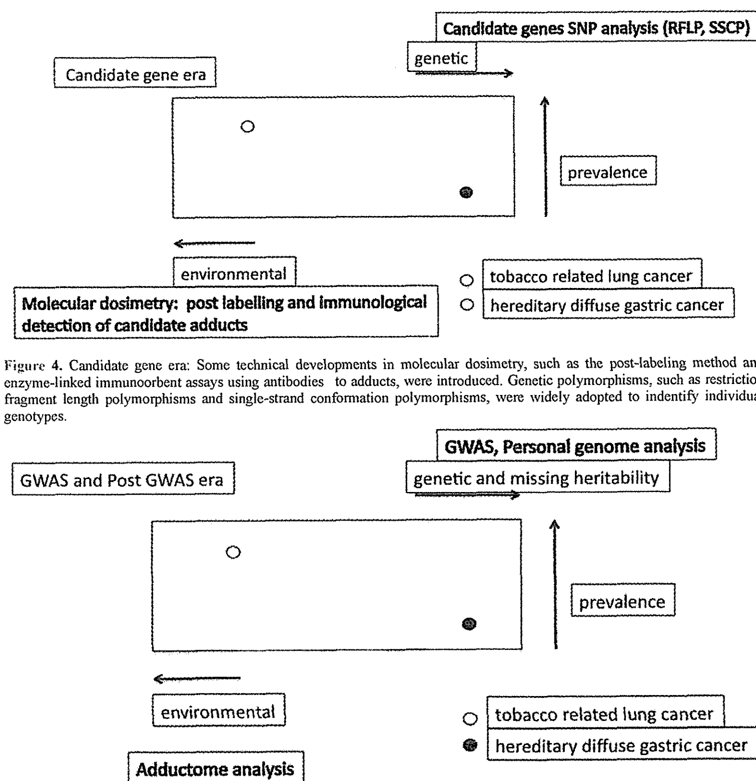

Figure 4. Candidate gene era: Some technical developments in molecular dosimetry, such as the post-labeling method and enzyme-linked immunoorbent assays using antibodies to adducts, were introduced. Genetic polymorphisms, such as restriction fragment length polymorphisms and single-strand conformation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

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