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

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(Received May 11, 2011/Revised July 5, 2011/Accepted July 10, 2011/Accepted manuscript online July 20, 2011/Article first published online August 19, 2011)

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* 2011; 102: 1782–1788)

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 Japa-

nese 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% CO₂ 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 frame-shift 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 μ g/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 \times SDS sample buffer and boiled. A 25- μ g 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).

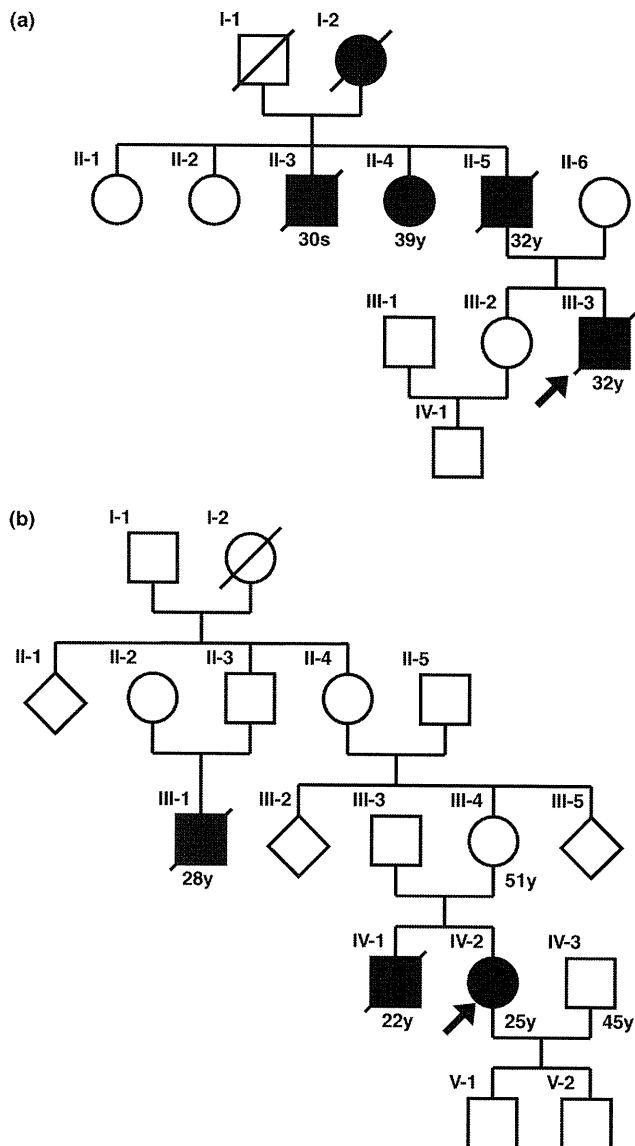


Fig. 1. Pedigrees of familial gastric cancer (FGC) families with germline *CDH1* alterations, specifically (a) a c.1212delC (p.Asn405IlefsX12) germline mutation of the *CDH1* gene and (b) a c.164-?,387+?del (p.Val55GlyfsX38) germline mutation of the *CDH1* gene. Squares indicate males; circles indicate 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⁾

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:

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

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.Asn405IlefsX12). 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).

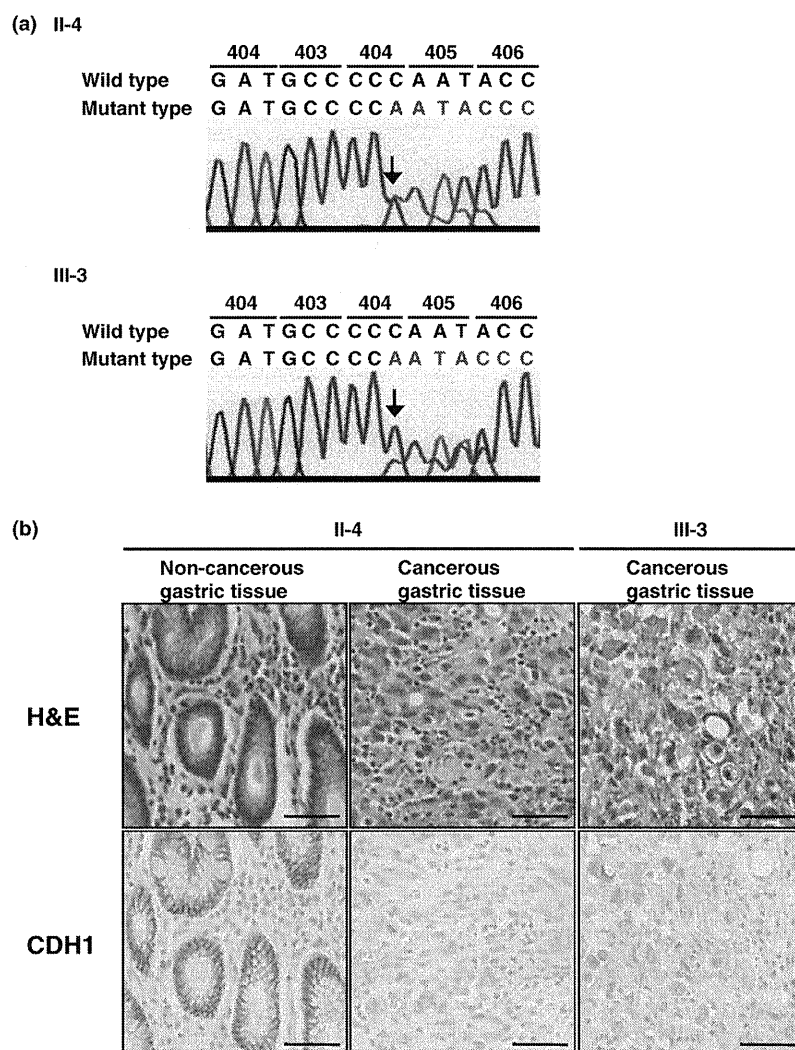


Fig. 2. Identification of the c.1212delC (p.Asn405IlefsX12) germline mutation of the *CDH1* gene. (a) Detection of the germline *CDH1* mutation in DNA from the male proband (III-3) and his aunt (II-4) using direct sequencing analysis. Both electropherograms of *CDH1* exon 9 show a heterozygous 1-bp deletion (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 μ m.

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

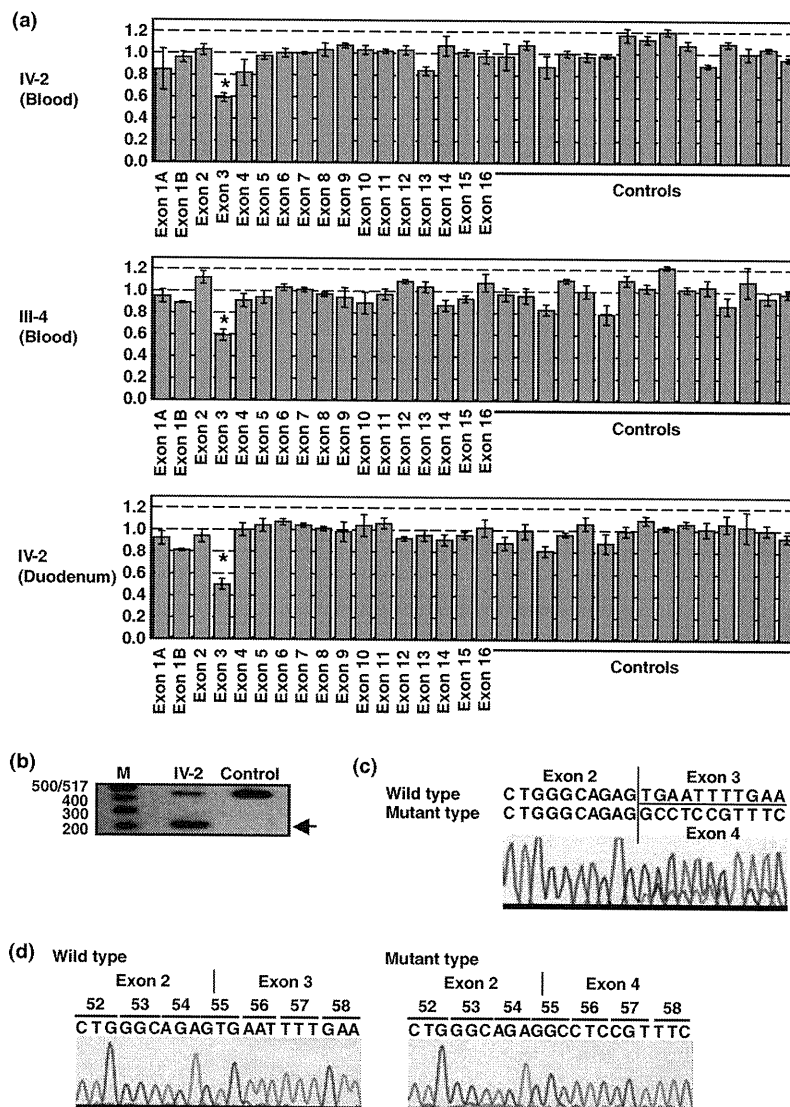


Fig. 3. Identification of large genomic deletion (c.164-?_387+?del) of the *CDH1* gene. (a) Detection of a decreased signal (asterisk) at *CDH1* exon 3 in DNA from the female proband (IV-2) and her mother (III-4) using multiplex ligation-dependent probe amplification (MLPA) analysis. The names of the MLPA probes are shown below the panels. Data are shown as the mean \pm 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.

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

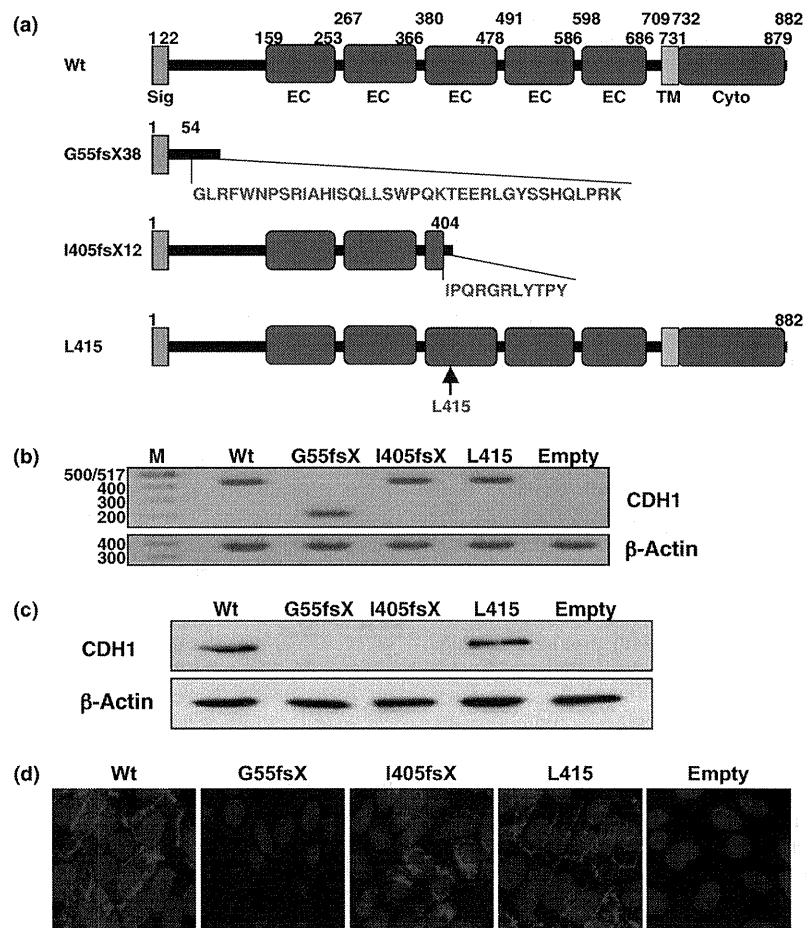


Fig. 4. Establishment of CHO-K1 cell lines stably expressing human CDH1 protein. (a) Scheme of the wild-type (Wt) and mutant (G55fsX38, I405fsX12, and L415) human CDH1 proteins. Amino acid sequences newly created by CDH1 genetic alterations are denoted in red in each mutant. Sig, signal peptide; EC, 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).

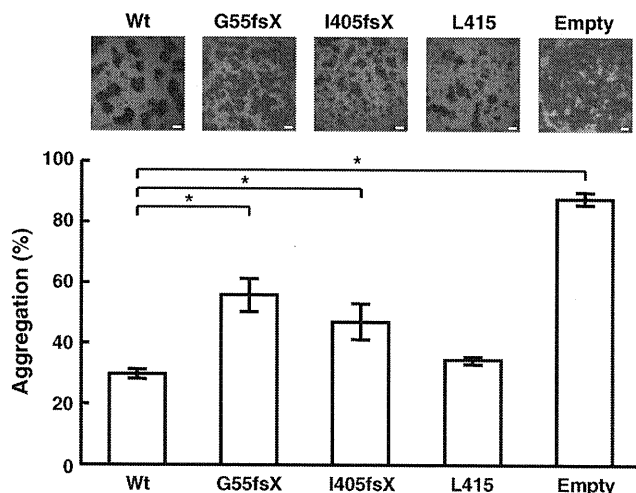


Fig. 5. Evaluation of the cell adhesion function of mutant-type CDH1 using a slow aggregation assay. Representative photographs of the results of a slow aggregation assay of CHO-K1 clones stably expressing wild-type (Wt) or mutant (G55fsX38, I405fsX12, and L415) human CDH1 are shown above the graph.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

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 pathogenic 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.

Acknowledgments

The authors acknowledge Mr T. Kamo (Hamamatsu University School of Medicine) for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare (21–1), the Japan Society for the Promotion of Science (21790383, 22590356, and 22790378), the Scientific Support Programs for Cancer Research, a Grant-in-Aid for Scientific Research on Innovative Area, the Ministry of Education, Culture, Sports, Science and Technology (221S0001), the Princess Takamatsu Cancer Research Fund, and the Smoking Research Foundation.

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

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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).

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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 methylation 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).

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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 susceptibility. 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 identified 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 rs10151730 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 rs10151730 locus, identified as rs402710 and rs2736100, which the investigators claimed, were independent, and the nearby genes were *TERT* and *CLPTMIL*, respectively. Another study, published at almost the same time, identified two loci at 6p21 and an additional polymorphism in the *CLPTMIL* 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

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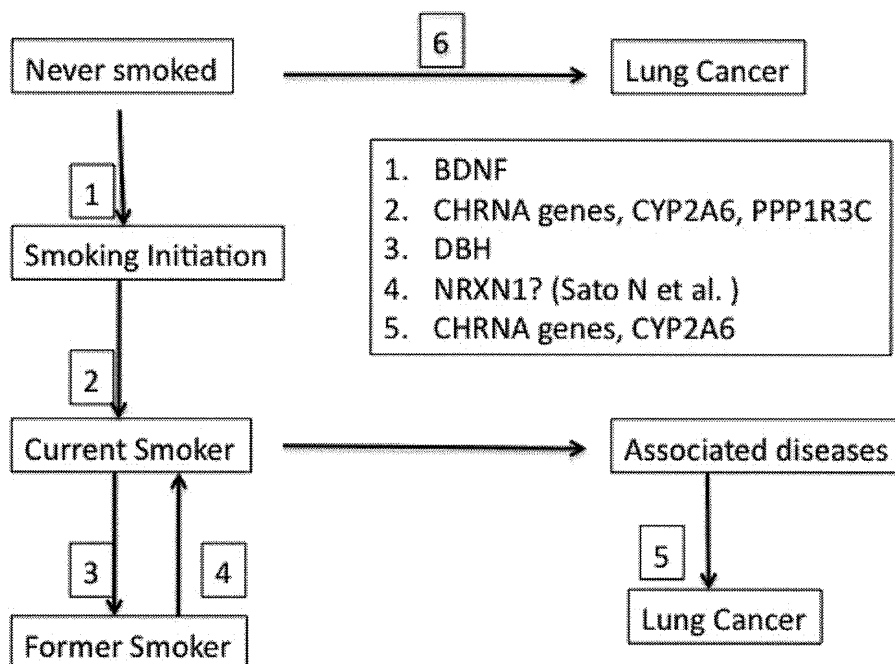


Figure 1. Presumed steps and genetic influences in the path from smoking to lung cancer. (Modified from (101). Amos CI, Spitz MR, Cinciripini P: Chipping away at the genetics of smoking behavior.) 1. Smoking initiation, 2. Smoking dosage, 3. Smoking cessation, 4. Difficulty quitting smoking, 5. Smoking 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 in 1963 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.

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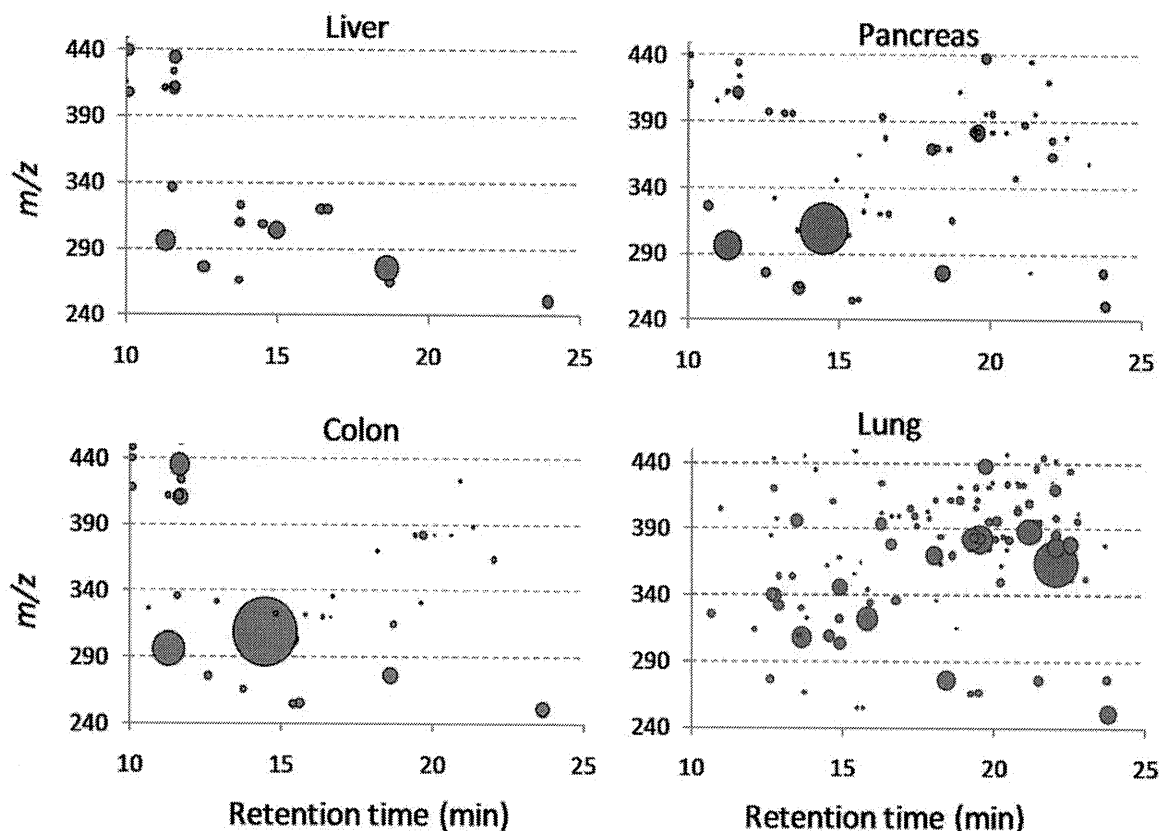


Figure 2. Adductome maps of 4 organs of the same individual. The position of each circle represent the species of the DNA adducts, and the size of each circle indicates the relative quantity of each DNA adduct. The adductome map shows that each organ contains more than one type of DNA adduct. The profile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

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DNA species in individual human tissues.

Table 1. Genetic polymorphisms most extensively evaluated for lung cancer susceptibility¹

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

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

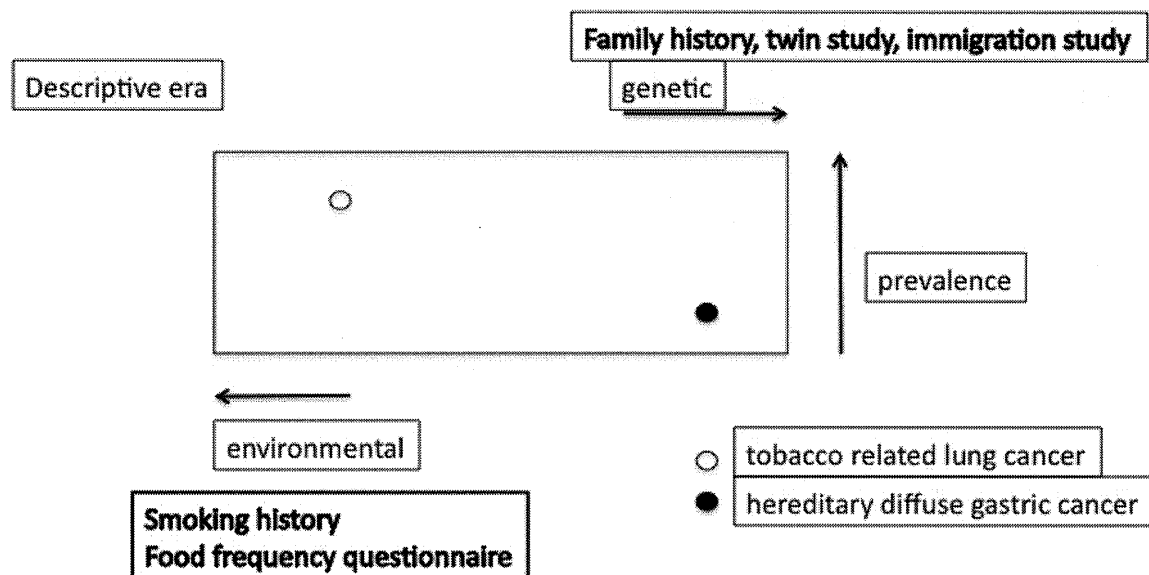


Figure 3. Methodological developments in nature and nurture analyses of cancer susceptibility. The arrows show how much environmental and genetic components influence the incidence of diseases. A disease like lung cancer (open circle) would be located in a more environmental position in the spectrum,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

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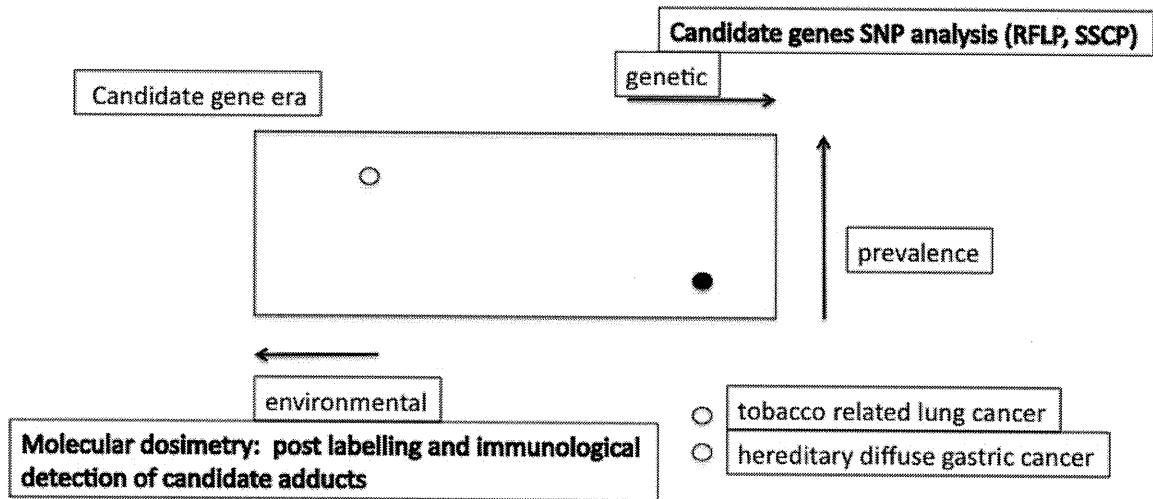


Figure 4. Candidate gene era: Some technical developments in molecular dosimetry, such as the post-labelling method and enzyme-linked immunorbent assays using antibodies to adducts, were introduced. Genetic polymorphisms, such as restriction fragment length polymorphisms and single-strand conformation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

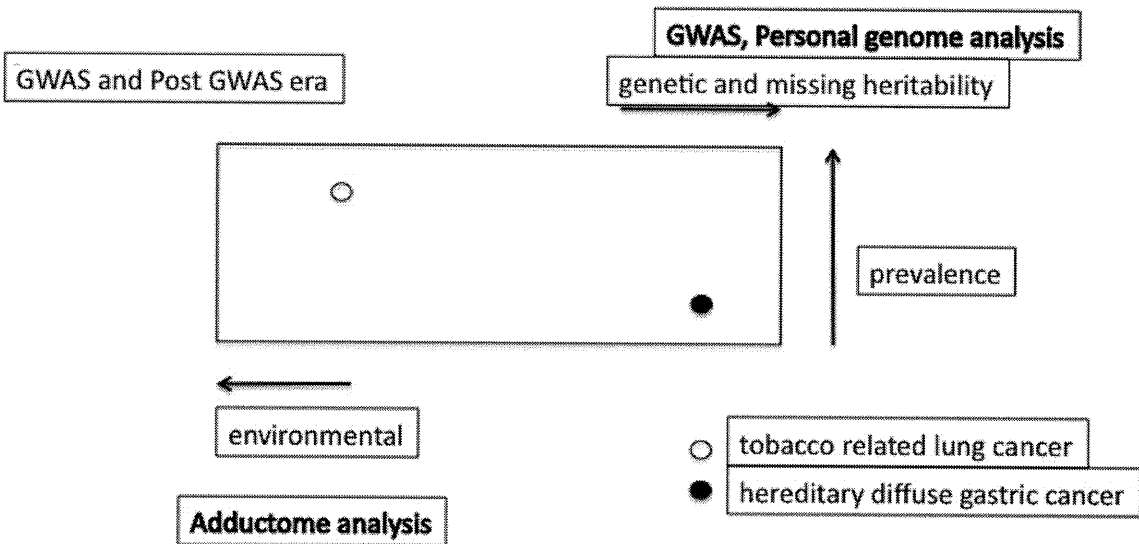


Figure 5. GWAS and post-GWAS era: The GWAS approach and post-GWAS approach (personal genome by next generation sequencing methods) are included among the methodologies aimed at the genetic aspects of lung cancer susceptibility. Adductome analysis emerged as a comprehensive method of detecting multiple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

8. ACKNOWLEDGEMENTS

This work was supported by Grants-in-

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Aid for Scientific Research (C) (22590356) and for priority areas (20014007 and 221S0001) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Grants-in-Aid for the 3rd Term Comprehensive 10-Year-Strategy for Cancer Control, for Cancer Research (21-1), and for Research on international cooperation in medical science from the Japanese Ministry of Health, Labour, and Welfare, and from the Smoking Research Foundation.

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