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Identification of disease biomarkers and screening of olfactory receptors for the biomarker detection

질병 바이오마커 발굴 및 그와 결합하는 후각 수용체 탐색

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Abstract

Identification of disease biomarkers and screening of olfactory receptors for the biomarker detection

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Due to the development of medical technology and systems, the premature mortality rate due to disease has decreased significantly compared to the past. However, lethality from some incurable diseases including cancer is still high. Because it is difficult to feel conscious symptoms before the disease develops to a late stage, and the existing diagnosis method is inaccessible due to the invasive method and cost of examination. Due to this reason, the latest disease diagnosis technology is developing in the direction of improving accessibility, and in particular, the need for non-invasive and economic method is emerging. As a typical example, the technology for diagnosing a disease by detecting a specific volatile organic compounds enables simple diagnosis without pain because it can detect the signal of disease from exhaled breath, sweat, urine, and saliva as well as blood and body fluids. In particular, the bioelectronic sensor has demonstrated excellent selectivity and sensitivity by combining a

primary transducer such as an olfactory receptor with a secondary transducer containing a nanostructured semiconductor such as carbon nanotubes or graphene.

The purposes of this research are identification of disease biomarkers and screening, performance evaluation of olfactory receptors for the detection of biomarkers that are essential for development of bioeletronic sensor. The selected diseases for study are lung cancer, tuberculosis, and gastric cancer. First, the discovery of biomarkers for lung cancer and the screening of human olfactory receptors were performed. The lung cancer cell line and the normal lung cell line were cultured to compare the composition of headspace gas by GC / MS, and volatile organic compound 2-ethyl-1-hexanol, which is more frequently generated in lung cancer cell lines, was identified. In addition, human olfactory receptors capable of detecting this biomarker were screened using a dual-glo luciferase reporter gene assay. It was confirmed that the identified olfactory receptor sensitively and selectively detects the lung cancer biomarker, and then conducted olfactory nanovesicle generation and performance evaluation for use as a primary transducer of the bioelectronic sensor in the further study.

In the second study, the screening of human olfactory receptors were carried out for identification of olfactory receptor capable of detecting 5 tuberculosis biomarkers found in urine [95]. The screening was conducted by transfectng the human olfactory receptor genes and the luciferase reporter gene into the HEK293 cell line to confirm the responsivity to the tuberculosis biomarkers. As a result, olfactory receptors recognizing each tuberculosis biomarker were selected, and their responsivity and selectivity were also analyzed.

Third, a number of exhaled breath samples of gastric cancer patients and healthy subjects were collected and analyzed using GC/MS. As a result, butyl acid and propionic acid, which are volatile organic compounds found in relatively large amounts in the exhaled breath of gastric cancer patients, were

identified. In particular, solid-phase microextraction (SPME) fibers were used as a instruments of collecting and concentrating volatile organic compounds to completely analyze the biomarkers containing a very small amount in the exhaled breath samples. To improve the reliability of the selected volatile organic compounds as biomarkers, we build a diagnostic model that distinguishes patients based on the amount of biomarkers in the exhaled breath through statistical analysis of overall data, and their sensitivity and selectivity were calculated. In addition, in order to identify a primary transducer of a bioelectronic sensor that detects biomarkers included in exhaled breath, the responsivity and selectivity of 2 human olfactory receptors known to detect butyric acid and propionic acid were estimated.

Development of disease diagnosis technology is an inevitable process for universal welfare and extension of life expectancy. Diagnostic methods targeting disease-specific volatile organic compounds are attracting attention in academia as a next-generation diagnostic technology, and are actively being studied all over the world. In this thesis, several disease-specific volatile organic compounds have been newly identified, and the human olfactory receptors capable of recognizing disease biomarkers were screened. The above research results are expected to be useful for the development of sensitive and selective bioelectronic sensor for disease diagnosis.

Key words: Volatile organic compound, biomarker, GC/MS, olfactory receptor, bioelectronic sensor

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List of Abbreviations

cAMP : cyclic adenosine monophosphate
CAR/PDMS : Carboxen/polydimethylsiloxane
CAR/PDMS/DVB : Carboxen/polydimethylsiloxane/divinylbenzene
CNT : carbon nanotube
CRE : cylic AMP-reponsding element
DMEM : Dulbecco's modified Eagle's medium
E. coli : Escherichia coli
FBS : fetal bovine serum
FET : field-effect transistor
GC/MS : gas chromathgraphy / mass spectrometry
GPCR : G-protein coupled receptor
HEK-293 : human embryonic kidney-293
LASSO : least absolute shrinkage and selection operator
OR : olfactory receptor
OSN : olfactory sensory neuron
PCR : polymerase chain reaction
PDL : poly-D-lysine
PDMS/DVB : Polydimethylsiloxane/divinylbenzene
RTP1S : receptor-transporting protein 1S
SEM : scanning electron microscope
SD : standard deviation
SPME : solid-phase microextraction
VOC : volatile organic compound

Chapter 1.

Research Background and Objectives

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Due to significant advance of medical technology, the average life expectancy of human was considerably prolonged and the fatal incurable diseases of the past are now curable. However, cancer and several other diseases are still one of the major causes of death for mankind, and especially for fatal diseases, rapid diagnosis is important to reduce mortality. As a typical example, cancer is a disease with a very high mortality rate at the end stage, although the symptoms of awareness are not clear at the early stage. Cancer accounts for 16 percent of all human deaths worldwide in 2016, and lung cancer is the leading cause of cancer death[1]. Early diagnosis is also important for highly infectious diseases. For example, tuberculosis is the world's top infectious disease, with 1.5 million people killed and 10 million infected in 2018. But because it is difficult for all people to visit medical sites frequently to diagnose diseases, point-of-care, remote diagnosis, and self-diagnosis have emerged as topics of next-generation diagnostic technology. What these methodologies have in common is that they pursue a fast, inexpensive, and non-invasive diagnostic process. Therefore, it is needed to replace the diagnostic methods that require large equipment and hygienic environment such as X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and biopsy.

A new diagnostic technology that has recently emerged is the analysis of disease-related volatile organic compounds (VOCs) from body fluids. The development of equipment, including gas chromatograph/mass spectrometer (GC/MS), has made it possible to analyze very low concentrations of chemicals, and to conduct sensory tests that were performed in the past based on individual sensory abilities in accordance with a unified standard. And it has been reported by researchers that the composition of VOCs originated from various body fluids, including exhaled breath, urine, and blood changes to reflect the condition of body. Some of these human-derived VOCs contain the potential as biomarkers of various diseases, and research is actively underway to explore the specific biomarker of diseases.

The methodology of detecting identified disease-related biomarkers is also an important topic. This is because very high sensitivity and selectivity are required for detection of biomarkers in body fluid. Thus, bio-inspired receptors were highlighted as candidates for the new primary transducers of disease diagnostic sensors to reinforce the selectivity aspects that lacked conventional electrochemical sensors. In particular, because olfactory receptors (ORs) are

specialized in detecting various VOCs, many attempts have been made to use them as a sensing material. However, it was only recently that the structure and mechanism of ORs began to be revealed. Since the function of many ORs have not been identified, it is necessary to screen the ligand through various biological experiments to use it for detection purposes.

The first objective of this research is identification of disease-related biomarkers from body fluids, including exhaled breath and headspace of cancer cells. The exhaled breath of gastric cancer patients and healthy controls were collected and analyzed by solid-phase microextraction (SPME)-GC/MS. The statistical analysis was conducted to construct a diagnostic model for discrimination of gastric cancer patients. The headspace gas of lung cancer cell lines and normal lung cell lines were also collected and analyzed by SPME-GC/MS. Next is screening of human olfactory receptors recognizing the biomarker of diseases. For this purpose, artificial olfactory cells produced by transfecting OR genes to HEK293 cells were used for screening of human ORs detecting lung cancer and tuberculosis biomarkers. Additionally, the responsivity and selectivity of ORs were assessed by intracellular calcium assay and luciferase reporter gene assays.

In summary, the objectives of this study are as follows:

1. Identification of lung cancer biomarkers by analyzing headspace gas of lung cancer cell lines and screening of human olfactory receptors recognizing the biomarker.

2. Screening of human olfactory receptors for detection of tuberculosis biomarkers in urine.

3. Identification of gastric cancer biomarkers by analyzing the exhaled breath of patients and healthy subjects before and after tumor resection.

In the thesis, the biomarkers for diagnosis of lung cancer and gastric cancer were identified. The human OR libraries were screened for detection of biomarkers of tuberculosis, lung cancer and gastric cancer.

Chapter 2.

Literature Review

Chapter 2. Literature Review

2.1 Volatolomics

Olfaction is a very important sense for living things to sense, judge, and respond to their surroundings. Most sensor technologies began by mimicking the olfactory function of living things, and thanks to advances in technology, sensors have now been developed that detect even low concentrations of chemicals that are difficult for humans to detect. Now, the research on volatile organic compounds (VOCs) has been conducted, with interest shifting to what substances should be detected and analyzed to be more useful and beneficial to human life. VOCs are generally referred to as low molecular weight organic compounds and are classified according to their emission source (e.g. material, building, indoor, outdoor, etc.), origin (e.g. endogenous or exogenous, biogenic or anthropogenic, etc.) and volatility [1]. In particular, it is known that VOCs generated from metabolomes including cells, tissues, and body fluids can change their composition and quantity by reflecting disorder, inflammation and oxidative stress in the body [2]. Based on these findings, volatolomics is a recently introduced approach to conduct disease diagnosis or health monitoring more easily and non-invasively than the conventional methods used in the medical field by analyzing biogenic VOCs. In general, volatolomics focuses on human body secretions including breath [3-5], saliva [6], urine [7], sweat [8], feces [9, 10], and has been studied extensively as a useful tool for disease diagnosis. However, because biogenic VOCs can occur in all living organisms, volatomics is gradually expanding its field of use [11-14].

2.2 Biomarkers of disease

Biomarker is a molecular indicator derived from biochemical pathways in living organisms [15]. Biomarkers are useful as a guideline for determining the state of disease and physiological state of an organism, for example, ribonucleic acid (RNA) [16], protein [17], antibody [18] and volatile organic compounds (VOCs) [19-21] are being studied as representative candidates for disease biomarkers. In order to find a biomarker for a particular disease, it is necessary to examine the body fluid of the patients. However, unlike non-invasive sources such as breath, urine, and feces, the collection process of blood and serum are invasive. Therefore, by culturing the target cancer cell line *in vitro* and analyzing its culture medium and headspace gas, it is possible to replace the body fluid analysis process that requires an invasive collection process [22, 23]. The biomarker-based disease diagnosis technology continues to expand its scope of application, and is expected to be used in clinical practice through clinical trials.

2.2.1 Volatile organic compounds related to disease

VOCs are being studied as candidates of biomarker for a variety of diseases due to the ease of the analytical process resulting from their volatility. Biomarkers of various diseases have been studied, including cancer [24-26], tuberculosis [27, 28], multiple sclerosis [29], and chronic kidney disease [30]. Representative methods used in the analysis process are gas chromatography/mass spectrometry (GC/MS) [31, 32], proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS) [33], selected ion flow tube mass spectrometry (SIFT-MS) [34] and solid-phase microextraction-GC/MS (SPME-GC/MS) [35, 36].

There are many types of VOCs that have been reported to be linked to disease. For example, in the exhaled breath of lung cancer patients, various alkanes [155], aldehydes [156-158], and alcohols [60, 73] showed potential as biomarkers. Fatty acids [25, 34, 61] have been reported as specific VOCs included in exhaled breath of patients with gastrointestinal and colorectal cancer, and ketones [159] have been identified as candidate biomarkers in urine of patients with prostate cancer. There are cases that the same VOC is selected as a biomarker for different types of diseases. This is because many diseases, including cancer and infectious disease, commonly induce inflammation and

oxidative stress in the body [71]. Therefore, it would be possible to identify the representative biomarker by cross-verifying VOCs generated by stress applied to organs and disease-related VOCs.

2.2.2 Sources and biochemical pathways of disease-related volatile organic compounds

VOCs detected in the human body can be derived from the outside or produced through various metabolic pathways in the cell and tissue. It is known that disease-related VOCs are produced mainly through pathological processes. Typically, VOCs are produced at a different ratio from normal conditions under the influence of metabolic disorders, oxidative stress, and genetic changes. The production process of disease-related VOCs is estimated based on the principles of cell biology, and various hypotheses about biochemical pathways have been suggested according to the types of VOCs reported so far.

For example, oxidative stress occurs under the influence of reactive oxygen species that are continuously produced in mitochondria, but can also occur from viral infections or exogenous sources such as cigarette smoke, pollution, and radiation. In this process, hydrocarbons such as volatile alkanes and methylated alkanes can be produced during the peroxidation of polyunsaturated fatty acids on the cell membrane and mixed with exhaled breath [160]. While normal cells produce ATP through oxidative phosphorylation through mitochondria, cancer cells tend to synthesize ATP by overactivating glycolysis process (Warburg effect). In cancer cells, glutaminolysis increases and the pentose phosphate pathway is promoted, producing a lot of nicotinamide adenine dinucleotide phosphate (NADPH) [161. 162]. In addition, pyruvate produced a lot through the overactivated glycolysis is converted to acetyl CoA by pyruvate dehydrogenase, and fatty acid synthesis is also highly increased in an environment where acetyl CoA and NADPH are sufficiently present [163]. Therefore, an increase of fatty acids nearby the cancer cells is observed in both cancer cell proliferation and carcinogenesis [164].

Various types of aldehydes have also been associated to lung cancer. It is known that hexanal and heptanal are contained in blood [119] and breath [168] of lung cancer patients, and formaldehyde is also reported to be more included

in the breath of lung cancer patients compared to the control group [157]. Studies have shown that propanal and butanal were also relatively more involved in exhalation in lung cancer patients [73]. Aldehyde can be produced in the body from alcohol due to the activation of alcohol dehydrogenase or cytochrome p450 (CYP2E1). The aldehyde dehydrogenase activity has been implicated in various biological and biochemical pathways and can be used to identify potential cancer stem cells [169]. It has been reported that aldehyde dehydrogenase 1L1 (ALDH1L1) is down-regulated in human liver, lung, ovary, pancreas, and prostate cancers [170]. This suggests that changes in the activity of aldehyde dehydrogenases related to tumor proliferation may affect the amount of aldehyde contained in breath.

It has been reported that the amount of acetone and 2-butanone also increase in exhaled breath of lung cancer patients [73]. Ketones are secondary products of lipid peroxidation, and it is speculated that an increase of ketone bodies is associated with a high oxidation rate of fatty acids, which are believed to be associated with weight loss in cancer patients [171]. However, ketone bodies also produced in the process of amino acid metabolism. In particular, it has been reported that exercise, fasting, and food consumption have an effect on the amount of acetone contained in exhalation [172, 173], suggesting that ketone possesses not only the potential as a biomarker for cancer cells, but also as a biomarker of stress applied to the body. Therefore, the potential of ketones as biomarkers of cancer still seems to be controversial.

Empirical research data on disease-related VOCs has been accumulated over the past decades. In addition to the simple disease-to-VOC association, many biochemical pathways in which VOCs are produced have been studied. There are still limitations in that research results remain in a state of possibility that does not coincide with a common direction. However, VOC study as a non-invasive disease biomarker is gradually increasing in value in terms of future diagnostic technologies, and its reliability is continuously improved due to the accumulation of research results and discovery of pathophysiological pathways.

2.3 Deorphanization and application of olfactory receptors

Olfactory receptor (OR) is located in the olfactory sensory neuron of the olfactory epithelium [37], and humans can distinguish more than 1 trillion odor combinations using about 400 functional ORs [38, 39]. Each ORs has its own ligand binding domain. And when the ORs were bound with a suitable odorants, the olfactory sensory neuron is activated to send electrical signals to the glomerulus, and these signals are transmitted to the brain to identify the smell [40]. The difference between ORs and sensing materials commonly used in conventional electrochemical sensors lies in their superior selectivity. Therefore, a bioelectronic sensor was developed that introduced G-protein coupled receptors [41], peptides [42], and ion channels [43] as primary transducers as well as ORs [44].

The bioelectronic sensor using the olfactory receptor as a sensing element is also called a bioelectronic nose. It has been reported that a bioelectronic nose can sensitively and selectively detect target odorants with a single carbon atomic resolution [45]. However, only a small number of ligands have been identified among the approximately 400 functional human ORs, and many studies are still underway to fully deorphanize human ORs. Experimental methods typically used for deorphanization of ORs using intracellular cAMP signaling pathway are calcium assay [46] and luciferase assay [47. 48]. The deorphanized OR proteins are coupled to field-effect transistor (FET) platform in the form of nanovesicles to be used as sensors for lung cancer diagnosis [49], environmental monitoring [50], food quality monitoring [51] and grain quality monitoring [52]. Previous studies about the performance and applications of bioelectronic nose were listed in table 2.1. Considering the combination of many odors that can be detected by humans, the field of application of bioelectronic nose is expected to expand in the future due to additional OR deorphanization.

Table. 2.1. Performance and applications of bioelectronic noses

Primary transducer		Secondary transducer	Odorant / ligand	Sensitivity	Application	Ref.
Type	Receptor					
Receptor-based	hOR2AG1	CNT	Amyl butyrate	100 fM	Fruit flavor	[45]
	hOR2AG1	CNT	Amyl butyrate	1 fM	Fruit flavor	[69]
	hOR2AG1	CPNT	Amyl butyrate	10 fM	Fruit flavor	[139]
	hOR2AG1, hOR3A1	Graphene	Amyl butyrate, Helional	0.1 fM	Fruit flavor, floral scent	[165]
	hOR3A1	CPNT	Helional	0.02 ppt	Floral scent	[166]
Cell-based	I7	Planar ele ctrode	Octanal	10 mM	-	[81]
Nanovesicle-based	hOR2AG1	CNT	Amyl butyrate	1 fM	Fruit flavor	[140]
	hOR1J2	CNT	Heptanal	10 fM	Lung cancer diagnosis	[84]
	cfOR5269	CNT	Hexanal	1 fM	Milk spoilage	[27]
	Receptor-derived peptide	CNT	Trimethylamine	10 fM	Seafood spoilage	[31]
	hOR51S1, hOR3A4	CNT	Geosmin, 2-methyl isoborneol	10 ppt	Water contamination	[50]
Nanodisc-based	hOR1A2	CNT	Geraniol, citronellol	1 fM	Rose scent	[167]

Chapter 3.

Experimental Procedures

Chapter 3. Experimental Procedures

3.1 Collection and analysis of headspace gas from cancer cell lines

3.1.1 Cell culture and headspace gas sampling

Three different cell lines were used: SK-MES (KCLB No. 30058), MRC-5 (KCLB No. 10171), human embryonic kidney 293 (HEK293). The SK-MES cells are derived from a human lung squamous cell carcinoma, and the MRC-5 cells are derived from a normal human lung fibroblast cell. The HEK293 cells were only used for the heterologous expression of human olfactory receptors (hORs) and the development of olfactory nanovesicles. These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (PS; Gibco, USA) at 37°C in a humidified atmosphere with 95% air/5% CO2. Before the headspace gas sampling process, SK-MES and MRC-5 cells were cultured to > 90% confluency in a 25 cm^2 T-flask.

We tried 3 type of SPME fibers including polydimethylsiloxane/ divinylbenzene (PDMS/DVB), carboxen/polydimethylsiloxane (CAR/PDMS), and carboxen/ polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB) for collection of volatile organic compounds in headspace gas of lung cancer cell line (all fibers were purchased from Supelco, USA). The chromatographic results are shown in figure 3.1. As a result, the PDMS/DVB fiber was the most absorbent as shown by the number of peaks which we were able to detect. Various SPME fibers, including polyacrylrate (PA), PDMS/DVB, CAR/PDMS, and CAR/PDMS/DVB have been used to collect VOCs from body fluids. Among them, it was reported that PDMS/DVB fiber showed good efficiency for the purpose of capturing the headspace gas component of cancer cell line [151], blood [152], urine [153], and mesenchymal stromal cells [154]. So, in this study, we considered that it would be efficient to use PDMS/DVB fiber to adsorb the VOCs of headspace gas of lung cancer cell line. The PDMS/DVB SPME fiber equipped with a manual SPME fiber holder (Supelco, USA) was exposed to headspace gas of cells for 17 hours. The culture flask was sealed with parafilm to avoid mixing headspace gas and outside air. For thermal

conditioning, the SPME fiber was heated at 250°C for 30 min before the sampling procedure.

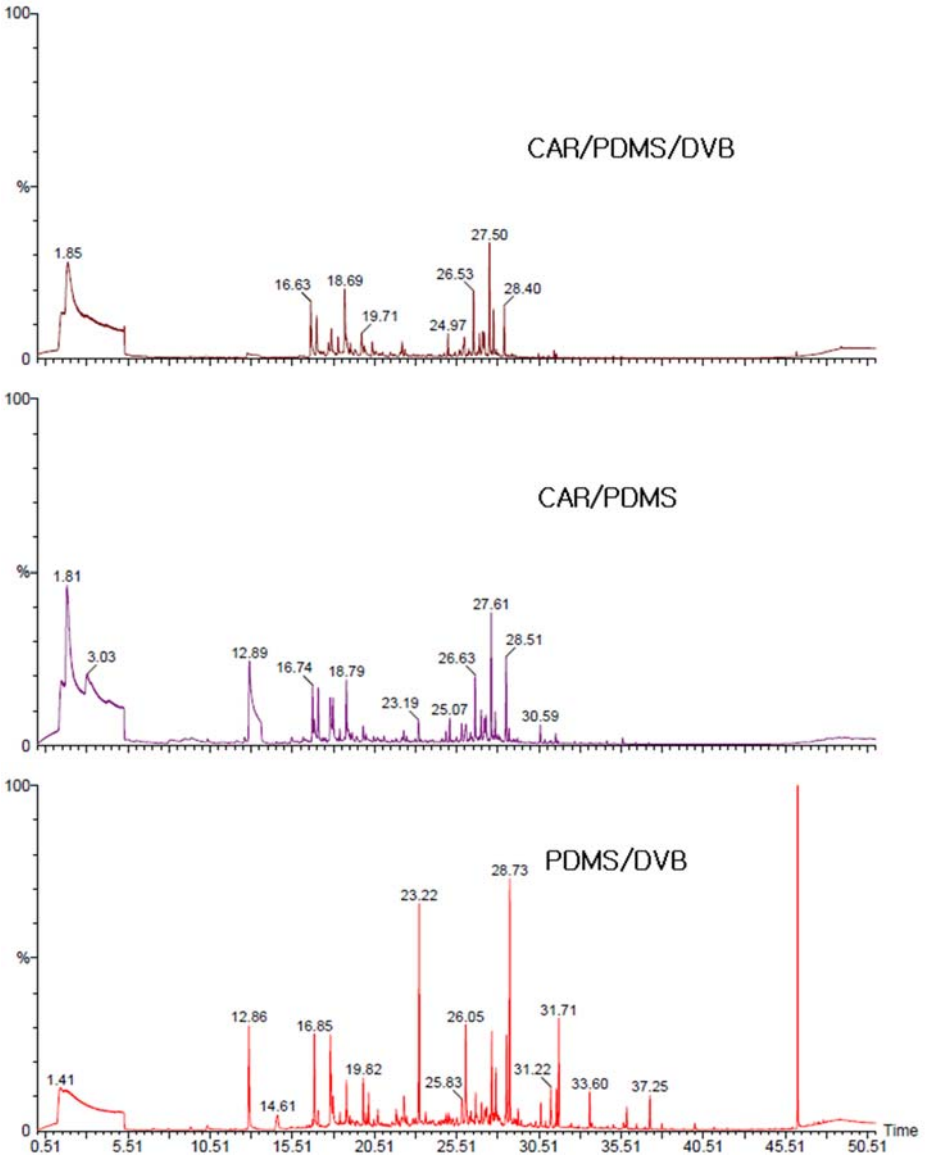

Figure 3.1. Comparison of efficiency of SPME fibers for collection of VOCs in headspace gas of lung cancer cell line.

3.1.2 Headspace gas analysis with GC/MS

The GC/MS analysis was performed on a Clarus 680 gas chromatograph coupled with a mass spectrometer Clarus 600T (both from Perkin Elmer, USA) equipped with HP-5MS SemiVol column (Agilent Technologies, USA). For identification of VOCs, the NIST 2012 library (NIST, USA) was used for a spectral match. After adsorption of headspace gas, the SPME fiber was injected into the GC inlet, which was pre-heated to 150°C for thermal desorption. The chromatography conditions for separation were as follows: initial oven temperature 35°C for 5 min, ramped up to 220°C at the rate of 5°C/min, further ramped up to 310°C at the rate of 15°C/min, and held steady for 3 min. The temperature of mass transfer line and ion source was 250°C. Chromatographic protocol for separation of VOCs collected from headspace gas of lung cancer and normal lung cell lines are described in Figure 3.2. The mass analyses were conducted in a total ion chromatography (TIC) mode with the full scan range of m/z = 20 to m/z = 300. For acquisition of chromatographic data, the Perkin Elmer Turbomass GCMS software (Perkin Elmer, USA) was applied.

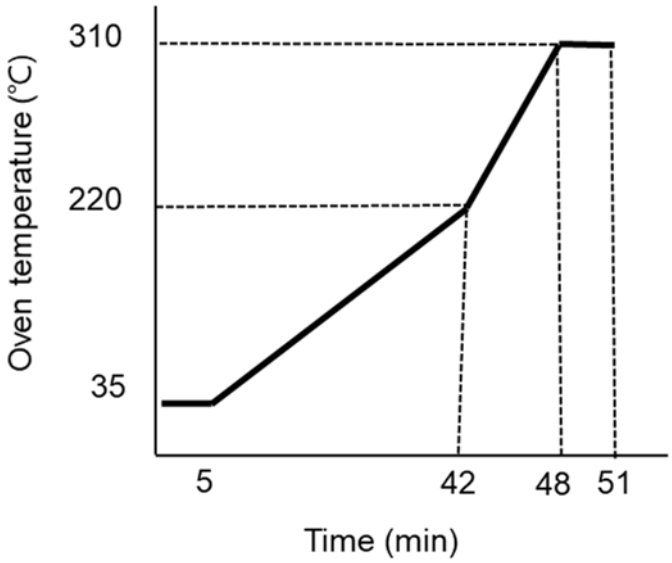

Figure 3.2. Chromatographic protocol for separation of VOCs in headspace gas of cell lines.

3.2 Identification of gastric cancer biomarkers from breath

3.2.1 Study groups and collection of clinical data

Gastric cancer patients and healthy subjects were recruited at the department of gastrointestinal surgery and the department of family medicine of Seoul National University Hospital (Seoul, Korea) for this study. The study protocol was approved by the Institutional Review Board of Seoul National University Hospital (IRB No: 1502-063-648). The inclusion criteria were the following: For the gastric cancer group, 1) pathologically diagnosed gastric adenocarcinoma at any age, 2) planned surgical treatment, and 3) informed consent. For the healthy control group, 1) planned gastric endoscopy for screening purposes, 2) no known history of any malignant disease, and 3) informed consent. The exclusion criteria, which were common to the patient and control groups, were as follows: 1) history of surgery for gastric cancer, 2) concomitant major respiratory diseases, 3) history of a primary cancer of any organ within the past 5 years, 4) pregnancy, and 5) an inability to participate in the study according to the judgement of the investigators.

Clinicopathologic data including age, sex, past medical history, current medications, history of smoking and alcohol consumption, and pathologic stage of gastric cancer was obtained by questionnaire and from medical records. In the healthy control group, absence of gastric cancer was re-confirmed by reviewing the result of the screening endoscopy. *Helicobacter pylori* (*H. pylori*) infection was assessed by one or a combination of studies including the campylobacter-like organism (CLO) test, urea breath test (UBT), *H. pylori* IgG serology, and/or the presence of *H. pylori* on the pathology report; patients were positive for *H. pylori* if any of these studies produced a positive result. Detailed information about the clinical characteristic of all study groups is shown in Table 3.1.

Table 3.1. Clinical characteristics of the study groups

Group	Sex	*N*	Age range (median)	Clinical stage				Gastric cancer risk factors		
				I	II	III	IV	Smoking status	Alcohol intake	*H. pylori* positivity
Gastric cancer	Total	64	25-77 (57)	18	14	26	6	11	17	5
	Males	44	32-77 (58)	7	11	22	3	11	15	1
	Females	20	25-76 (56)	11	3	4	3	0	2	4
Healthy subject	Total	61	24-77 (52)	-	-	-	-	8	26	2
	Males	30	33-77 (55)	-	-	-	-	7	18	1
	Females	31	24-66 (51)	-	-	-	-	1	8	1

3.2.2 Sampling of exhaled breath and environmental gas

Prior to exhaled breath sampling, all patients fasted for at least 8 hours. The subjects' oral cavity was washed with water. All subjects rested for at least 10 min while inhaling ambient air in a selected space in the hospital before breath collection. Afterwards, exhaled breath was collected in a 1 L Tedlar bag (BMS, Japan). Samples were taken at 3 time points for each gastric cancer patient - before the surgery, 1 week after surgery, and more than 1 month after surgery. The exhaled breath sample collected 1 week after surgery reflects the effects of tumor resection. Samples collected more than 1 month after surgery reflect the patient's condition after the inflammatory response has been alleviated. Environmental gas was also collected each time exhaled breath was collected. Sampling of the breath of the healthy control group was done in the same manner before the screening endoscopy. Cosmetic usage could not be prohibited for all the individuals, so information about their usage of cosmetics was collected.

3.2.3 SPME-GC/MS analysis

All breath samples were analyzed within 6 h after collection. Polydimethylsiloxane-divinylbenzene (PDMS-DVB) and carboxen-polydimethylsiloxane (CAR-PDMS) coated 75 μM SPME fibers (both from Supelco, USA) and manual SPME holders (Supelco, USA) were thermally cleaned for 30 min at 300°C before analysis. The SPME fiber was injected into the Tedlar bag through a silicon cap to prevent leaking and contamination, and exposed to the exhaled breath sample for 45 min at room temperature. GC/MS analysis was performed on a Clarus 680 gas chromatograph coupled with a Clarus 600T mass spectrometer (both from Perkin Elmer, USA) equipped with a VF-624 ms column (Agilent Technologies, USA). The column length was 60 m and its diameter was 530 μm. The film thickness was 3.0 μm. The SPME fiber was injected into the GC inlet, which was pre-heated to 250°C for thermal desorption. The chromatographic program for separation was as follows: initial oven temperature, 35°C, held for 5 min, then ramped up by 5°C/min to 150°C, again ramped up by 10°C/min to 250°C, and held for 5 min. The temperature of the mass transfer line and the ion source were 250°C. The mass analysis was done in total ion chromatography (TIC) mode with full scan range of m/z = 20 to m/z = 300. Turbomass GCMS software (Perkin Elmer, USA) was used for

acquisition of chromatographic data. The NIST 2012 mass spectral library (National Institute of Standard and Technology, USA) was used for spectral matching and identification of VOCs.

3.2.4 Statistical analysis

All statistical analyses were conducted with SAS version 9.3 (SAS institute, USA), R (version 3.6.2, R Foundation for Statistical Computing, 2019), and Prism 7.0 (Graphpad Software, USA). A p-value $\leq$ 0.05 was taken to indicate statistical significance. The Mann-Whitney test was applied to compare the peak area (abundance) of VOCs in the exhaled breath of gastric cancer patients and healthy subjects as obtained by SPME-GCMS analysis. The least absolute shrinkage and selection operator (LASSO) regression method was applied to construct an appropriate diagnostic model for gastric cancer due to the multicollinearity of analysis results [53]. The accuracy of the diagnostic model in distinguishing patients from controls was determined by using the area under the curve (AUC) of receiver operating characteristic (ROC) curves.

3.3 Gene cloning

The hOR2W1 and cfOR0312 coding sequences were amplified by polymerase chain reaction (PCR) from human genomic DNA mixture and canine genomic DNA mixture, respectively. The PCR was conducted, with 0.5 mM of each primer, PCR-premix including Taq polymerase, and 100 ng of human and dog genomic DNA. Temperature protocol of PCR followed this cycle: 35 cycles of 95℃ for 5 min, 54℃ for 30 s, and 72℃ for 1 min. The sizes of PCR products, were confirmed by gel electrophoresis. The Rho-tag sequence (N-MNGTEGPNFYVPFSNKTGVV-C) was fused with N-terminus of hOR2W1 and cfOR0312 gene, and inserted to multiple cloning sites of the pcDNA3 vector using a DNA ligation kit.

3.4 Production of olfactory receptor proteins

3.4.1 Expression of olfactory receptors in mammalian cells

HEK293 cells were grown in DMEM culture medium, containing 10% FBS and 1% PS at 37℃ in 96 well plates, and a humidified environment with 95% air / 5% CO_2. The Rho-tag and DYKDDDDK-tag fused hOR, $G\alpha_{olf}$, receptor transporting protein 1S (RTP1S) genes were all subcloned to pcDNA3 mammalian expression vectors (Invitrogen, USA). The type-3 muscarinic acetylcholine receptor (M3-R) gene was subcloned into pCI mammalian expression vector. The HEK293 cells were harvested and resuspended in the resuspension buffer R (Invitrogen, USA) to a density of 3.0×10^6 cells mL^{-1}. For the development of olfactory nanovesicles, 100 µl of cell solution was mixed with 5 µg of hOR, 1 µg of $G\alpha_{olf}$, and 0.5 µg of RTP1S. Using the Neon-Transfection System (Invitrogen, USA), the transfection was conducted by applying 10 ms electric pulses three times at 1100 V to the gene-cell mixture solution. Transfected cells were cultured in 10% FBS-containing DMEM solution without any antibiotics.

In case of utilizing lipofectamine 2000 tranfection reagent, transfection was conducted with a mixture of 150 µl DMEM, 0.5 µg Lipofectamine 2000, 0.2 µg pcDNA3 plasmids including OR genes at 70-90% confluency of cells per well. These cells were incubated for 6 h in transfection mixture, at 37℃, and replaced to 100 µl of culture medium.

3.4.2 Generation of olfactory nanovesicles

After 48 h culture following transfection, the hOR-expressing HEK293 cells were washed with Dulbecco's phosphate-buffered saline (dPBS, Gibco, USA) and incubated in DMEM containing 10 μg mL^{-1} of cytochalasin B (Sigma, USA) with agitation at 300 rpm and 37°C for 30 min. The procedure of generation of olfactory nanoveiscles are shown in Figure 3.3. For separation of developed nanovesicles from the parent cells, the incubated cells were centrifuged at 1000g for 10 min in Eppendorf tubes, and the supernatant was subsequently centrifuged at 15000g for 30 min. The separated nanovesicles were resuspended in dPBS with 1000 ng mL^{-1} of total protein and stored at -70°C, and were melted before use.

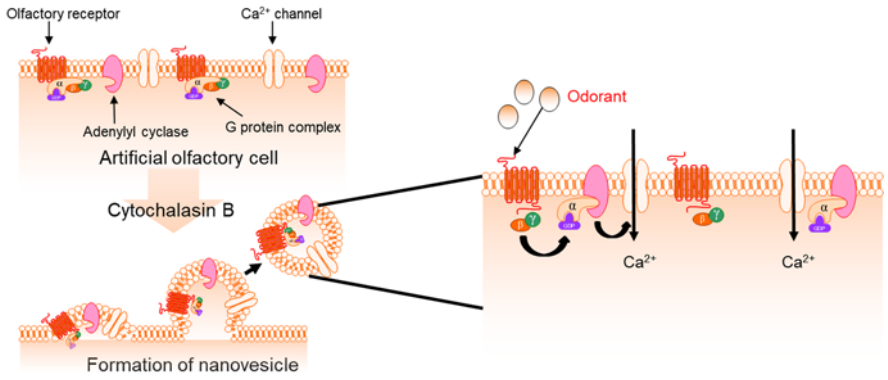

Figure 3.3 Schematic diagram of olfactory signaling and generation of olfactory nanovesicles.

3.5 Characterization of olfactory receptor proteins

3.5.1 Immunocytochemistry

For identification of hOR2W1 and cfOR0312 expression on HEK293 cells, *rho*-tag antibody was diluted to 5 μg/ml in PBS, as a primary antibody solution, and anti-rabbit IgG Alexa fluor 594-conjugated antibody was diluted to 4 μg/ml in PBS, as a secondary antibody solution. For identification of OR2Z1, OR4M1, OR2A1, OR5H1 and OR5C1, anti-rabbit Alexa fluor 488-conjugated antibody was used as secondary antibody. Transfected cells were fixed using 4% paraformaldehyde solution in PBS for 20 min, and treated with 1% BSA solution in PBS for 1 h at 37℃. After that, fixed cells were incubated in primary antibody solution for 90 min and sequentially incubated in secondary antibody solution for 90 min. Cell pictures were captured, after 5 times washing by PBS solution.

3.5.2 Western blot analysis

Nanovesicles in dPBS solution were mixed with 5X SDS sampling buffer and loaded onto a 10% SDS page gel. Next, the proteins were transferred to a PVDF membrane (Bio-Rad, USA) under 0.15 A current for 1 h. The membrane was incubated in blocking solution (TBS solution mixed with 0.1% Tween-20 and 5% skim milk) at room temperature for 1 h, followed by incubation of the membrane overnight with TBS mixed with 0.1% Tween-20 and 5% BSA and 0.1% DYKDDDDK tag antibody (Cell Signaling Technology, USA). The membrane was washed three times for 5 min with TBS mixed with 0.1% Tween-20, and was incubated in TBS mixed with 0.1% Tween-20, 5% BSA, 0.1% anti-rabbit IgG-HRP (Invitrogen, USA) at room temperature for 1 h. For detection of membrane proteins, ECL solution (Thermo Scientific, USA) was used.

3.5.3 Calcium signaling assay

The hOR-expressing HEK293 cells were washed with PBS, and incubated in Ringer's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, 1 g/L Pluronic® F-127 with 5 μM

of Fura-2 AM at 37℃ and pH 7.4 for 30 minutes. After intake procedure of Fura-2 AM by HEK293 cells, the medium was exchanged with Ringer's solution with 10 μM of probenecid, and incubated at 37℃ for 1 h. This procedure reduces leakage of Fura-2 AM from cells, by blocking organic anion transport with probenecid [54]. And then, real-time measurement of fluorescence intensities was conducted with GENios Pro microplate reader. Excitation light was applied at 340 and 380 nm, and emission wavelength was recorded at 510 nm.

The calcium signaling assay protocol for olfactory nanovesicles is as follows: The hOR-expressing HEK293 cells were incubated in Ringer's solution [140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM glucose, 10 mM HEPES (pH 7.4)] with 10 μM of Fura-2 AM (Invitrogen, USA) at 37°C for 30 min. Next, following previously described procedure, the nanovesicles including fura-2 AM were developed from cells. The intracellular calcium-induced fluorescence was measured at 510 nm emission wavelength and at dual excitation wavelengths of 340 nm and 380 nm using TECAN Genios Pro (TECAN, Switzerland). The schematic diagram of calcium signaling assay is described in Figure 3.4.

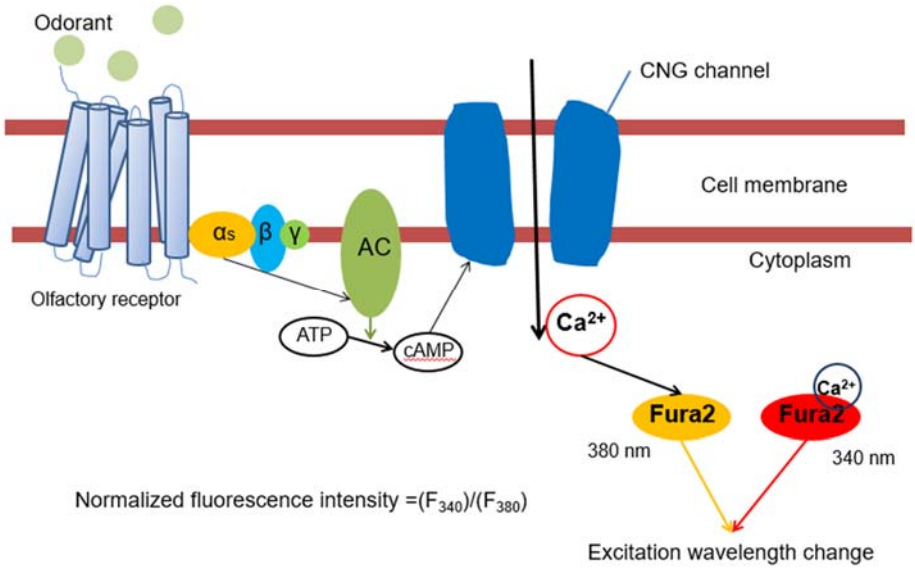

Figure 3.4 Schematic diagram of intracellular calcium signaling assay.

3.5.4 Dual-glo luciferase assay

The hOR and luciferase reporter gene-expressing HEK293 cells were cultured for 24 h after transfection with 2.5×10^5 cells per well in 96-well plates. Subsequently, the culture medium was replaced with 50 µl DMEM per well and incubated for 30 min at 37°C in a humidified atmosphere with 95% air/5% CO_2. After incubation, 25 µl of diluted odorant solution was added to each well and incubated at 37°C for 4 h. The odorant-evoked luciferase response of transfected cells was measured with a Dual-Glo® Luciferase Assay System (Promega, USA) and Luminoskan Ascent Microplate Luminometer (Thermo Scientific, USA). The activity of firefly and Renilla luciferase was normalized with the following formula:
[Firefly / Renilla (N) – Firefly / Renilla (0)] / [Firefly / Renilla (FSK) – Firefly / Renilla (0)].

For measurement of positive cAMP-evoked response and negative control response, 1 µM of forskolin (FSK) and 1% DMSO diluted in DMEM were added without any odorants. Prism 7.0 (Graphpad Software, USA) and Sigmaplot 12.0 (Systat Software, USA) was used to draw graphs and for statistical analysis.

Chapter 4.

Identification of a lung cancer biomarker using a cancer cell line and screening of olfactory receptors for biomarker detection

Chapter 4. Identification of a lung cancer biomarker using a cancer cell line and screening of olfactory receptors for biomarker detection

4.1 Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death. In 2018, lung cancer accounted for 11.6% of the total cases and 18.4% of the total cancer deaths [55]. Therefore, the diagnosis of lung cancer is a serious challenge because of its lethality, and several studies explored the possibility of developing a better diagnostic tool. Many types of diagnostic methods are used in clinical practice. For example, chest X-ray [56], magnetic resonance imaging (MRI) [57], computed tomography (CT) [58], and biopsy [59] are currently used for diagnosis of lung cancer. However, the diagnostic methods currently used in the medical field are often invasive or require expensive equipment, and thus an improved diagnostic method is required. A new technology for disease diagnosis based on volatile organic compound (VOC) monitoring is the focus of interest, currently. Rapid development of analytical tools like gas chromatography/mass spectrometry (GC/MS) has shown that substances released from the human body may represents signs of disease.

Sources of VOCs derived from the human body can be distinguished into external and internal factors. Food intake and inhalation of environmental air represent external sources of VOC, whereas cell metabolites, body wastes, and secretions are internal sources. A disease-related disorder alters the amount and composition of metabolites in the body, which is likely to be reflected in substances discharged from the human body through the circulatory system. Cancer cells showing excessive metabolism compared with normal cells also stress the surrounding organs, and tend to produce additional metabolites.

Exhaled breath can include tumor-related VOCs mixed with pulmonary air. Therefore, analysis of the exhaled breath of cancer patients is a promising technique for the discovery of novel biomarkers [60-62, 34]. However, in the case of exhaled breath, it is difficult to specify the increased biomarker levels because they are mixed with various endogenous and exogenous VOCs excreted from the lungs through the esophagus and mouth. Therefore, a direct analysis of headspace gas of cancer cells has been reported [22, 23, 63]. The

headspace gas contains medium and volatile components of the cell metabolite. Therefore, in a well-controlled in vitro environment, headspace gas is considered to represent an optimized sample for the analysis of the metabolic output of cells with minimal external influence. The equipment mainly used for gas analysis is GC/MS, and solid-phase microextraction (SPME) fiber has been used to collect VOCs in the headspace gas [64-66].

On the other hand, sensor technology for detecting a disease-specific biomarker has also been developed. In addition to the existing electrochemical sensor technology, due to the discovery of nano-scale devices, a sensitive semiconductor sensor technology for bioanalytical applications using carbon nanotube (CNT) or graphene has been reported [67,68]. The fusion of biologically derived elements like olfactory receptors and taste receptors to act as primary transducers on the basis of electrochemical sensors is known as a bioelectronic sensor, which has recently been studied using various devices [42, 69, 70]. The sensory receptors are attracting attention as a potent transducer for bioelectronic sensor that compensate for the lack of selectivity in chemical sensors.

The present study was conducted to identify the lung-cancer specific biomarker by comparing VOC profiles in headspace gas of cancer cell lines and controls. The SPME fiber was used to collect the headspace gas, and GC/MS was adopted as an analytical device. After the identification of biomarker, human olfactory receptor library was screened to find a proper receptor for the detection of a lung cancer biomarker. Selected olfactory receptor was expressed in HEK-293 cells and nanovesicles containing the olfactory receptor. In order to utilize the selected olfactory receptor as a primary transducer of a bioelectronic seccnsor to be developed later, the olfactory receptor-containing nanovesicles were constructed.

4.2 Collection and analysis of headspace gas of lung cancer cell line

The overall procedure for headspace gas collection and analysis is schematically described in Figure 4.1. The lung cancer cell line SK-MES and the normal lung fibroblast cell line MRC-5 were selected as sources of VOCs. The optical images of cell lines during the culture are shown in Figure 4.2. The VOCs in the headspace gas of cells were adsorbed on the PDMS/DVB fiber overnight, and analyzed by GC/MS.

The chromatographic profiles of VOCs collected from the headspace gas of culture medium, MRC-5 cells, and SK-MES cells are shown in Figure 4.3. Since all of them were cultured in the same medium, the profiles of VOCs were generally similar. However, a peak showed a distinct quantitative difference, which confirmed that the peak detected at the retention time (RT) of 18 min was significantly higher in the headspace of SK-MES cells (Figure 4.4) than in MRC-5 cells and culture medium. As a result of mass spectrometric analysis, this peak was identified as 2-ethyl-1-hexanol, a VOC previously reported as a potential biomarker candidate for diagnosis of lung cancer [71].

Alcohols in human body not only originate in food intake, but are also derived from the metabolism of hydrocarbons. The alkanes are hydroxylated by the cytochrome p450 enzymes, which are induced during carcinogenesis, to several types of alcohols [72]. For example, 1-propanol was found at higher concentrations in the breath of lung cancer patients than in healthy subjects [73]. According to a previous study, the concentration of 2-ethyl-1-hexanol was also increased in the headspace gas of NCI-H2087, another lung cancer cell line, compared with medium control [74]. In addition, 2-ethyl-1-hexanol was specifically found in the saliva of lung cancer patients compared with other cancers [75]. These results support the idea that specific alcohols represent potential biomarkers of lung cancer.

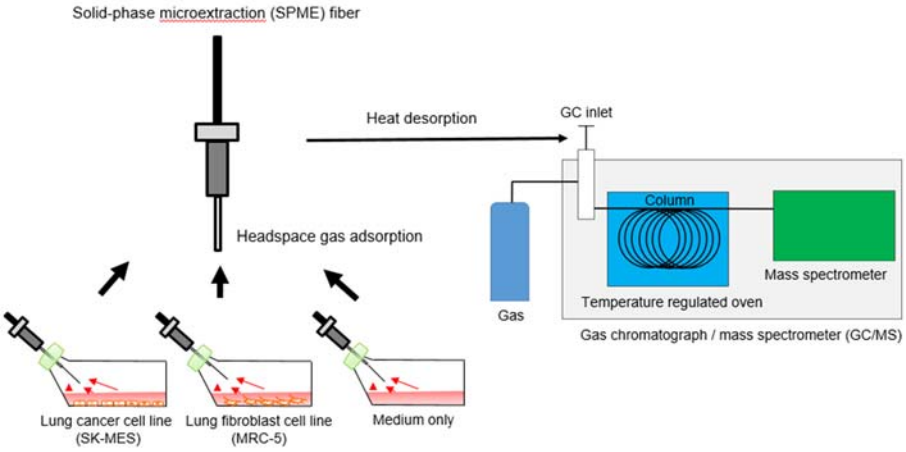

Figure 4.1. Schematic diagram of headspace gas analysis with SPME-GC/MS.

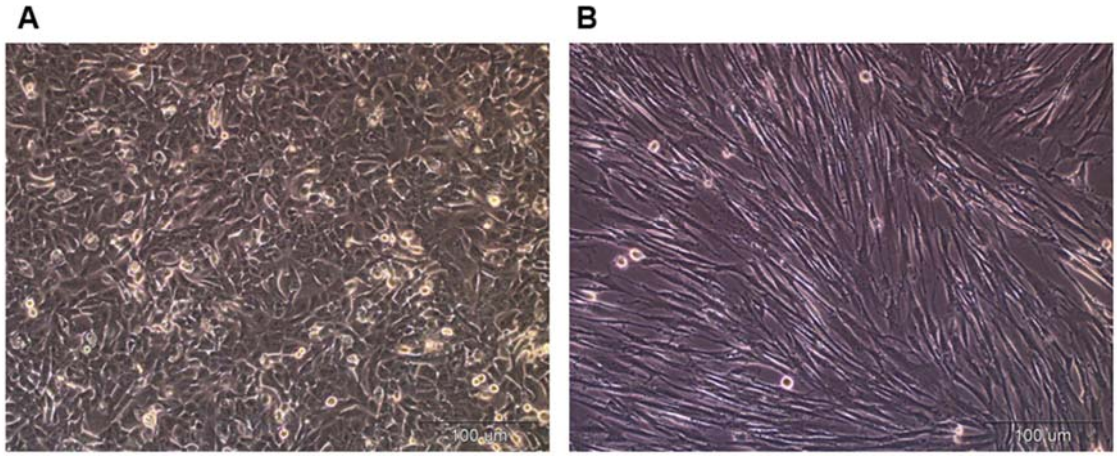

Figure 4.2. Optic image analysis of (a) SK-MES and (b) MRC-5 cell lines.

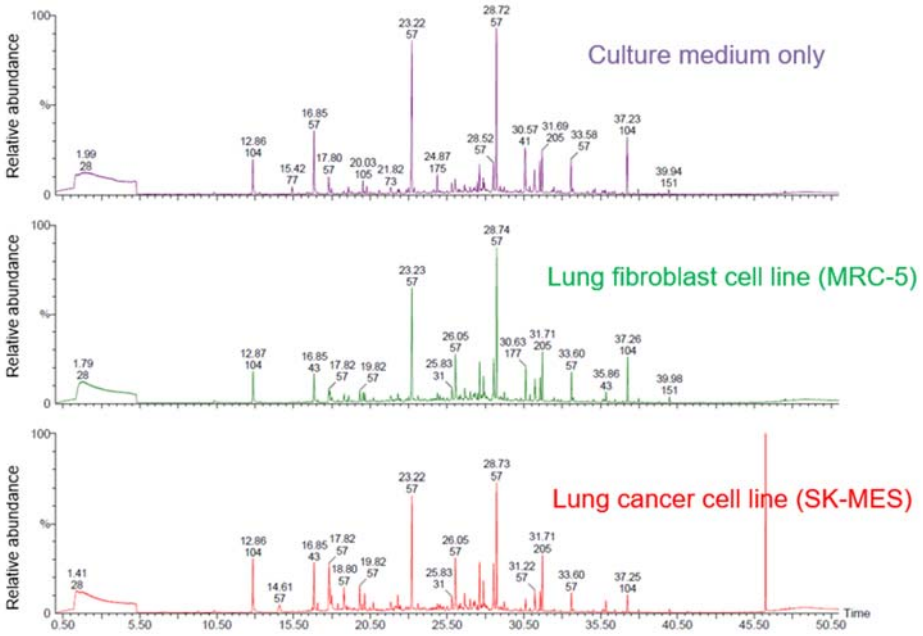

Figure 4.3. Chromatographic results of headspace gas collected from SK-MES, MRC-5 and culture medium.

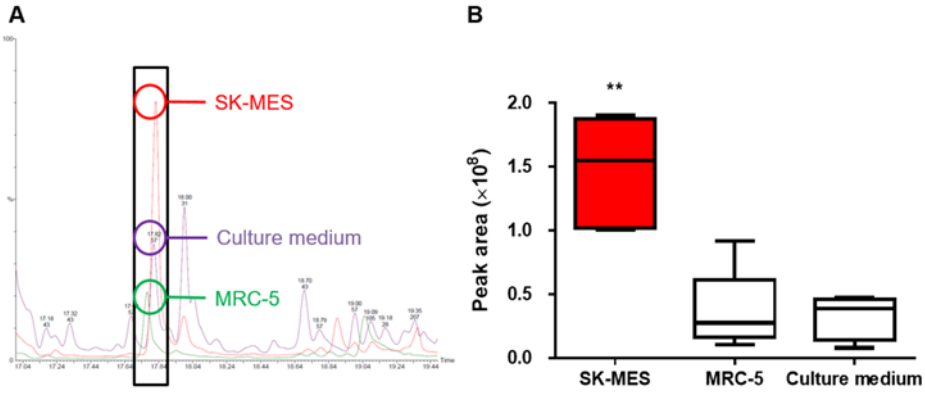

Figure 4.4. Identification of lung cancer-specific VOC by analyzing VOC profiles of headspace gas of SK-MES, MRC-5 and culture medium. (A) Overlay of 3 chromatograms (B) Comparison of the peak areas (arbitrary unit) at RT 17.80 min (n=5). The non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (Significance of results, ** : $p < 0.01$).

4.3 Screening of human olfactory receptors recognizing 2-ethyl-1-hexanol

Although researchers have investigated the function of olfactory receptors, many factors still remain unknown. For deorphanization of receptors, several cellular assays such as intracellular calcium signaling assay [76, 77] and luciferase assay [50, 78] were adopted. These methods were developed based on the intracellular signaling transduction pathways of olfactory receptor expressed in a heterologous system [79]. To identify a sensory receptor capable of detecting 2-ethyl-1-hexanol, the human olfactory receptors were investigated. In this study, 145 different human olfactory receptor genes were inserted into a pcDNA3 mammalian expression vector, and the Dual-Glo luciferase assay was conducted with pCI mammalian expression vector including cAMP responding element (CRE) reporter gene. The human olfactory receptor-expressing HEK293 cells were exposed to 100 μM 2-ethyl-1-hexanol. Based on receptor screening, OR4D11P was identified as the most sensitive receptor for 2-ethyl-1-hexanol among the 145 human olfactory receptors (Figure 4.5A). Because of the characteristic metabolites produced by lung cancer cells, there is a high possibility of contamination with various VOCs in breath or serum, so the selectivity of the receptor is an important issue. By comparing the responsivity to 100 μM hexanal, heptanal, 2-ethyl-1-hexanal, 2-ethyl-1-butanol, and 2-ethyl-1-hexanoic acid, it was found that OR4D11P exhibits robust selectivity to 2-ethyl-1-hexanol (Figure 4.5B). Since the olfactory receptor discriminates the target molecules based on the structural characteristics of the ligand-binding site [80], it is encouraging that the structurally similar chemicals are strongly distinguished by OR4D11P.

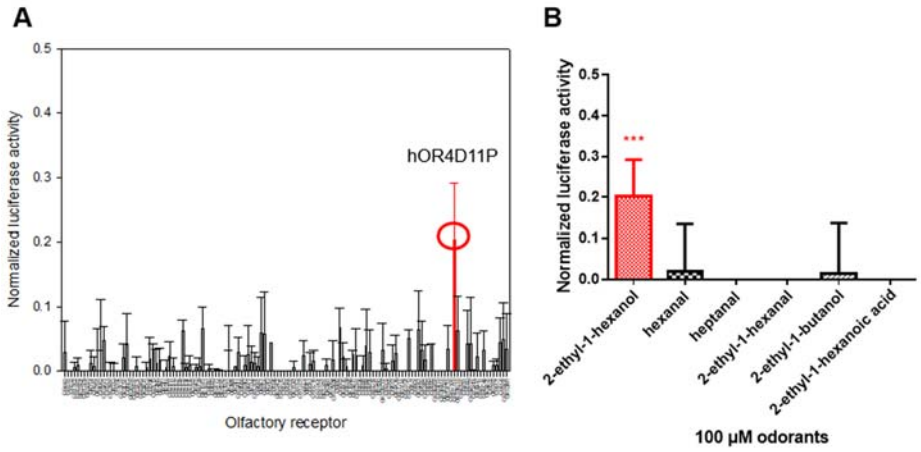

Figure 4.5. (A) Screening result of 145 human olfactory receptors exposed to 100 μM 2-ethyl-1-hexanol. (B) Selectivity test of OR4D11P exposed to 100 μM 2-ethyl-1-hexanol with structurally similar odorants. The error bar indicates standard deviation (n=6). The non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (Significance of results, *** : $p < 0.001$).

4.4 Generation and characterization of olfactory nanovesicles

Several studies utilized whole receptor-expressing cells as sensing elements to detect VOCs [81, 82]. However, since the whole cell is a very large and unstable sensing element, studies have been conducted to develop a biological transducer that is smaller and simpler to immobilize while preserving the sensory performance of the receptor. A typical example is a cell-derived nanovesicle [83]. In particular, olfactory nanovesicles express olfactory receptors [51, 84]. In this study, the olfactory nanovesicles containing OR4D11P were produced as sensors to detect lung cancer biomarkers. Western blot analysis confirmed the robust expression of olfactory receptor proteins in the nanovesicles produced by treating HEK293 cells with cytochalasin B (Figure 4.6A). In addition, a calcium signaling assay was used to confirm that the olfactory receptor proteins contained in these nanovesicles detected 2-ethyl-1-hexanol similar to their expression on the cell surface. As shown in Figure 4.6B, the nanovesicles containing OR4D11P responded significantly to 1 mM 2-ethyl-1-hexanol compared with those without olfactory receptors.

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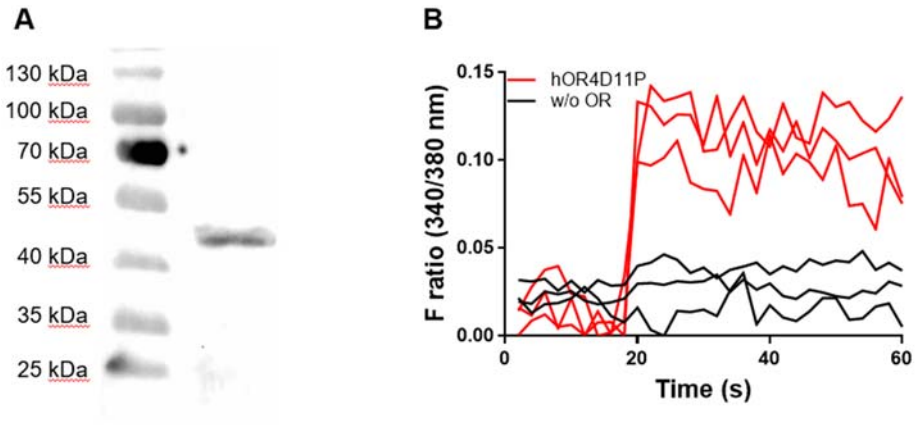


Figure 4.6. Characterization of olfactory nanovesicles. (A) Western blot of OR4D11P expressed on HEK293 cells. (B) Calcium signaling assay of OR4D11P-expressing nanovesicles and empty-vector transfected nanovesicles exposed to 1 mM 2-ethyl-1-hexanol.

4.5 Conclusions

The VOC profiles of headspace gas of SK-MES, MRC-5 and medium control were compared via SPME-GC/MS analysis to identify a lung cancer-specific biomarker. As a result, we found that the concentration of 2-ethyl-1-hexanol was significantly higher in the headspace gas of SK-MES than in MRC-5 and medium control. This odorant is a potential lung cancer biomarker, which has been reported several times in similar studies conducted earlier. To identify the sensory receptor for the detection of 2-ethyl-1-hexanol, 145 human olfactory receptors were expressed on the HEK293 cell surface and screened via Dual-Glo luciferase assay. After the screening procedure, OR4D11P was identified as a sensitive and selective receptor to 2-ethyl-1-hexanol. To utilize this receptor in stabler and smaller area than the whole cell, the olfactory nanovesicles were fabricated using cytochalasin B. Western blot analysis and calcium influx assay confirmed that OR4D11P was strongly expressed on the nanovesicle and the sensing ability was also retained. These results are expected to play a role in developing biomaterial-based sensors for the diagnosis of lung cancer.

Chapter 5.

Screening of human olfactory receptors to detect tuberculosis-specific volatile organic compounds in urine

Chapter 5. Screening of human olfactory receptors to detect tuberculosis-specific volatile organic compounds in urine

5.1 Introduction

Tuberculosis (TB) is highly contagious disease. In 2018, 10 million new TB patients were reported, and 1.5 million people died from tuberculosis [85]. Due to advances in hygiene and medical technology, the incidence of new tuberculosis patients decreases by approximately 2% each year. But it is difficult to identify carriers of TB because the initial symptoms are similar to those of the common cold. Therefore, the importance of diagnostic technology has been emphasized. However, most current TB diagnosis methods involve an invasive process. For example, tuberculin skin test [86] and interferon-gamma release assay [87] are representative tuberculosis diagnostic methods currently performed in the medical field. Because these methods all include an injection process, they require skilled medical personnel and a clean environment in the treatment process. So there has been a steady increase in demand for non-invasive methods of tuberculosis diagnosis.

In order to meet these expectations, the biomarker diagnostic method is currently attracting attention as a new non-invasive tuberculosis diagnostic method. Microbial markers in sputum [88, 89], urine tuberculosis DNA [90], urine lipoarabinomannan [91], and volatile organic compounds (VOCs) [92, 93] were reported as candidates for new tuberculosis biomarkers [94]. VOCs derived from the body contain various metabolic products, and are known to be a clue to infer information on diseases occurring in the body. Among them, urine is a sample that can collect volatile biomarkers non-invasively. According to a study by Banday *et al*., a blind test using a diagnostic model based on the quantitative variation of 5 biomarkers detected in the urine of tuberculosis patients was able to distinguish tuberculosis patients at 98.8% sensitivity [95]. Various sensors have been developed to detect VOCs, but it is difficult to selectively detect specific biomarkers in the case of conventional electrochemical sensors.

For this reason, bioelectronic sensor technology was developed to enhance selectivity by integrating bio-derived receptors into electrical sensors [50,69]. The primary transducer mainly used in bioelectronic sensors is the olfactory

receptor (OR) that has sensory detection as its main function, and several studies have been conducted to reveal its sensing function [37, 76]. However, since many ORs still remain in the orphan status, it is necessary to find a suitable receptor to detect VOCs. Therefore, we will identify ORs that can detect TB biomarkers for the future development of a bioelectronic sensor.

5.2 Screening of human olfactory receptors

Dual-glo luciferase assay was performed to identify the human olfactory receptor (hOR) detecting TB biomarkers. First, in order to confirm that HEK293 cells do not show a nonspecific response to biomarkers, the responsivity of HEK293 cells transfected with empty pcDNA3 mammalian expression vector to 1 mM concentration of cymol, 2,6-dimethylstyrene, isopropyl acetate, o-xylene and 3-pentanol was assessed (Figure 5.1). As a result, it was confirmed that HEK293 cells did not react non-specifically to TB biomarkers.

A total of 135 hORs were screened, and it was confirmed that OR5H1, OR2A1, OR2Z1, OR5C1, OR5M1 could detect cymol, 2,6-dimethylstyrene, isopropyl acetate, o-xylene, and 3-pentanol, respectively (Figure 5.2). The responsivity between each ORs and the ligand was compared with the signal size when a mixture of 5 tuberculosis biomarkers was added to HEK293 cells not expressing OR, and all 5 receptors responded well to each ligand (Figure 5.3).

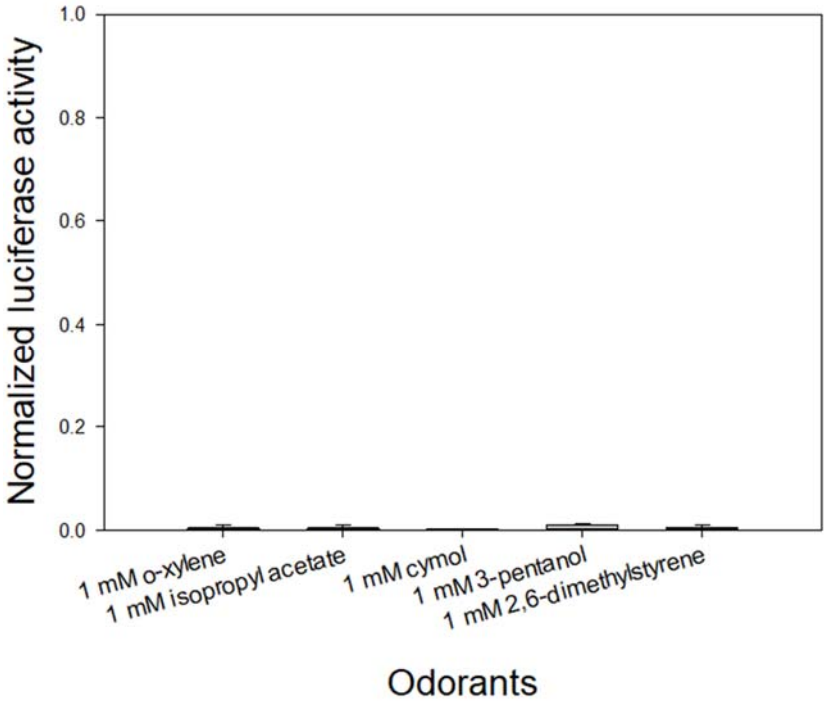
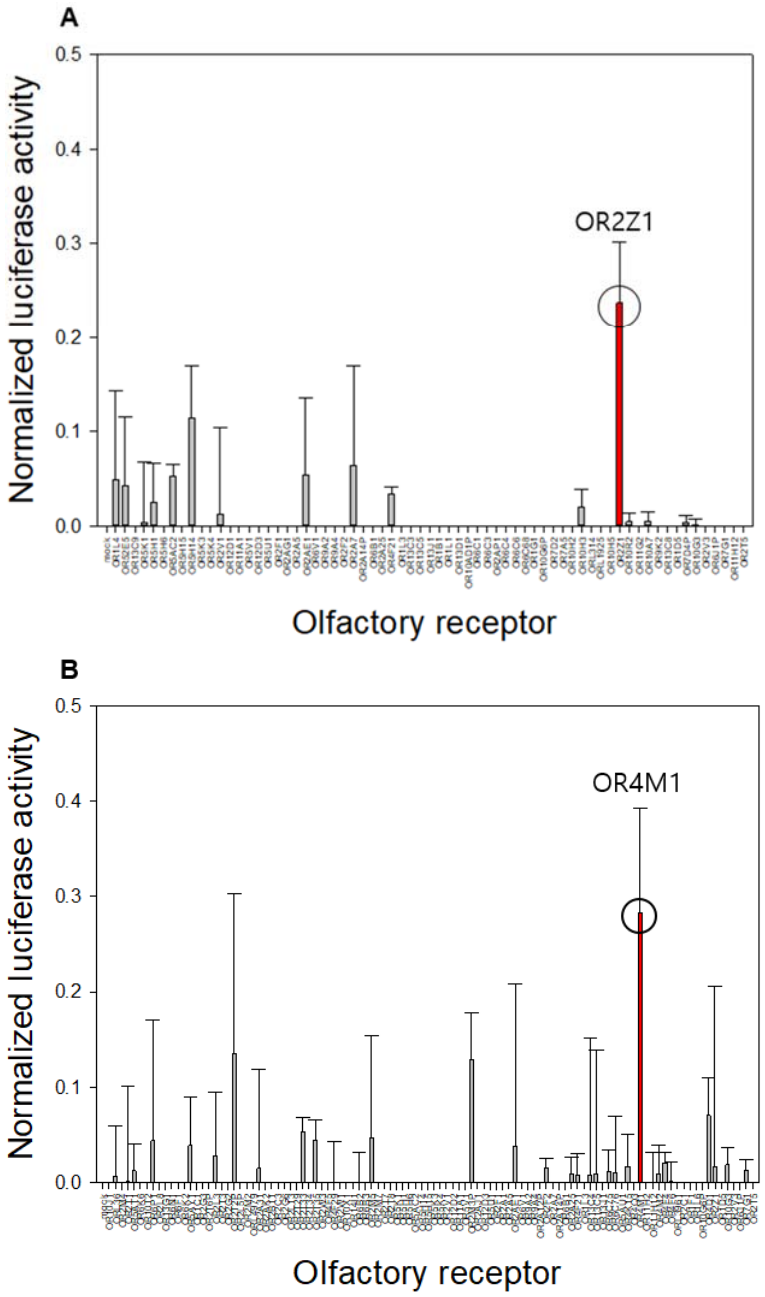


Figure 5.1. Response of HEK293 cells without ORs to tuberculosis (TB) biomarkers.


A
Normalized luciferase activity
0.5
0.4
0.3
0.2
0.1
0.0
OR2Z1
Olfactory receptor
B
Normalized luciferase activity
0.5
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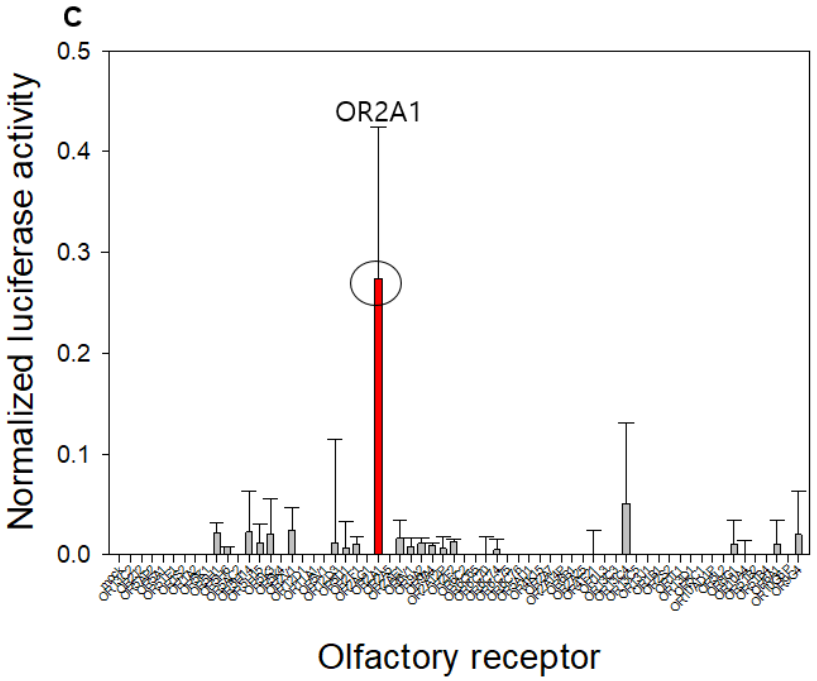
0.0
OR4M1
Olfactory receptor


C
Normalized luciferase activity
0.5
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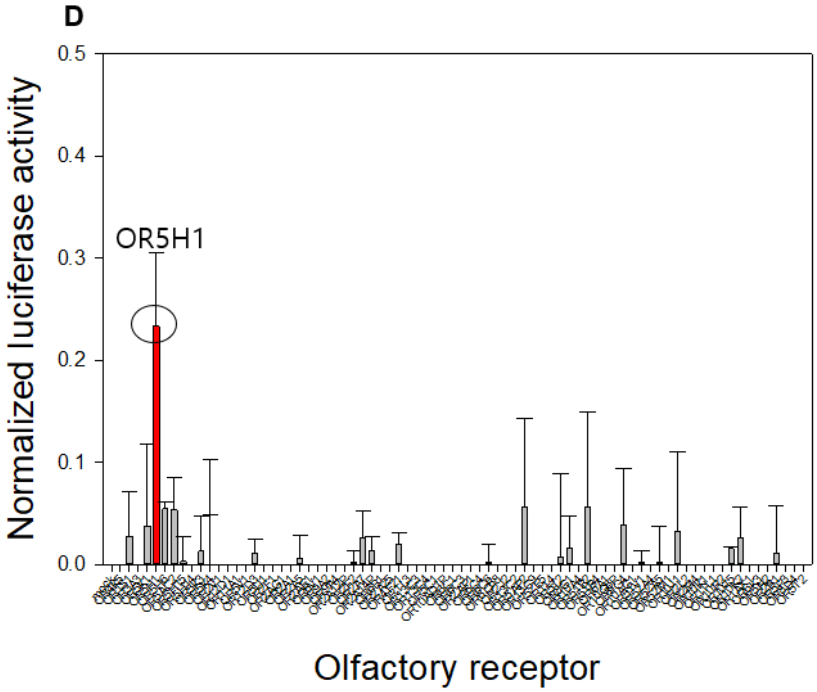
0.0
OR2A1
Olfactory receptor


D
Normalized luciferase activity
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OR5H1
Olfactory receptor

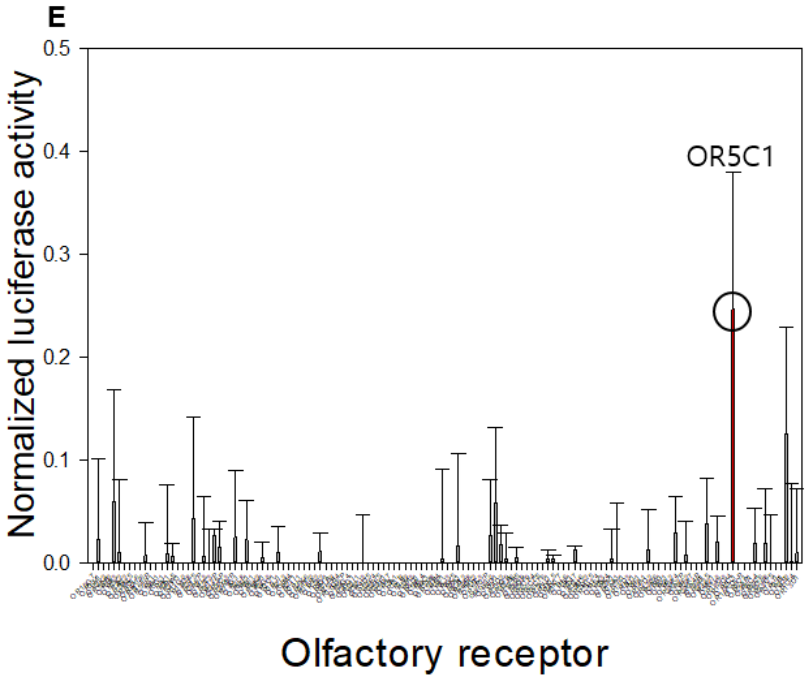

Figure 5.2. Screening results of human olfactory receptors (hORs) to TB biomarkers. Normalized luciferase activities of (A) 68 hORs to cymol, (B) 108 hORs to 2,6-dimethylstyrene, (C) 64 hORs to isopropyl acetate, (D) 82 hORs to o-xylene, (E) 135 hORs to 3-pentanol were measured by Dual-glo luciferase assay method.

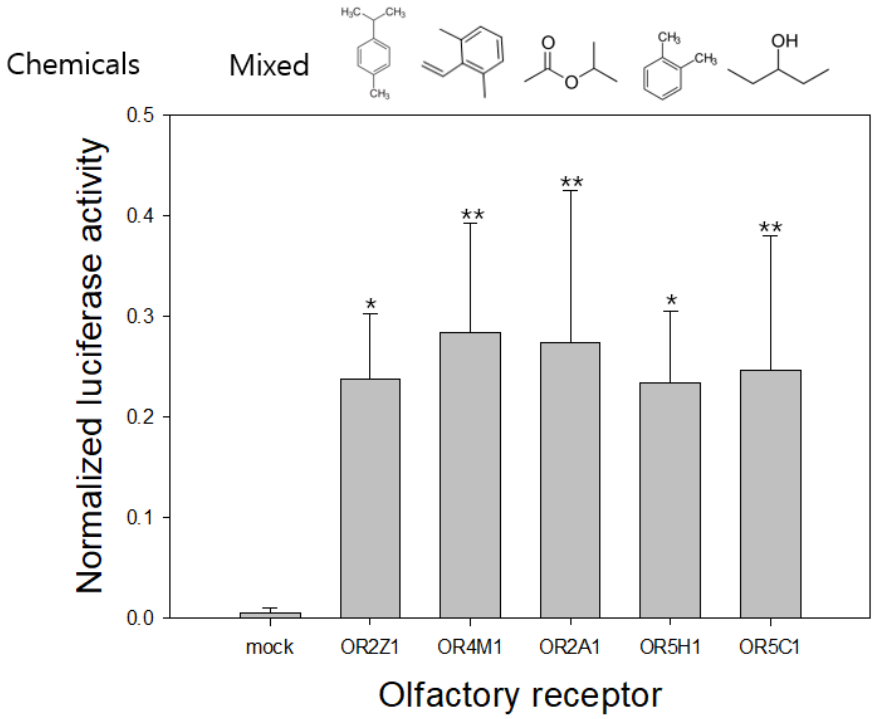

Figure 5.3. Responsivity of screened hORs to 1 mM ligands - OR2Z1 to cymol, OR4M1 to 2,6-dimethylstyrene, OR2A1 to isopropyl acetate, OR5H1 to o-xylene, OR5C1 to 3-pentanol - were compared to responsivity of non-transfected HEK293 cells to mixed TB biomarkers. A non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (significance of results, * : $p < 0.05$, ** : $p < 0.01$).

5.3 Characterization of olfactory receptors recognizing biomarkers of tuberculosis

Immunocytochemistry was performed to confirm that the identified 5 hORs were well expressed on membrane of HEK293 cells. As shown in Figure 5.4, 5 hORs were well expressed in membrane of HEK293 cells. In addition, in order to confirm the selectivity of identified hORs to each ligand, responsivity of 5 hORs to 1mM mixture of TB biomarkers excluding ligand was examined. As a result, it was confirmed that OR2Z1, OR2A1, and OR5C1 selectively respond to cymol, isopropyl acetate, and 3-pentanol. On the other hand, OR5H1 and OR4M1 showed a slight cross-responsivity with other tuberculosis biomarkers (Figure 5.5).

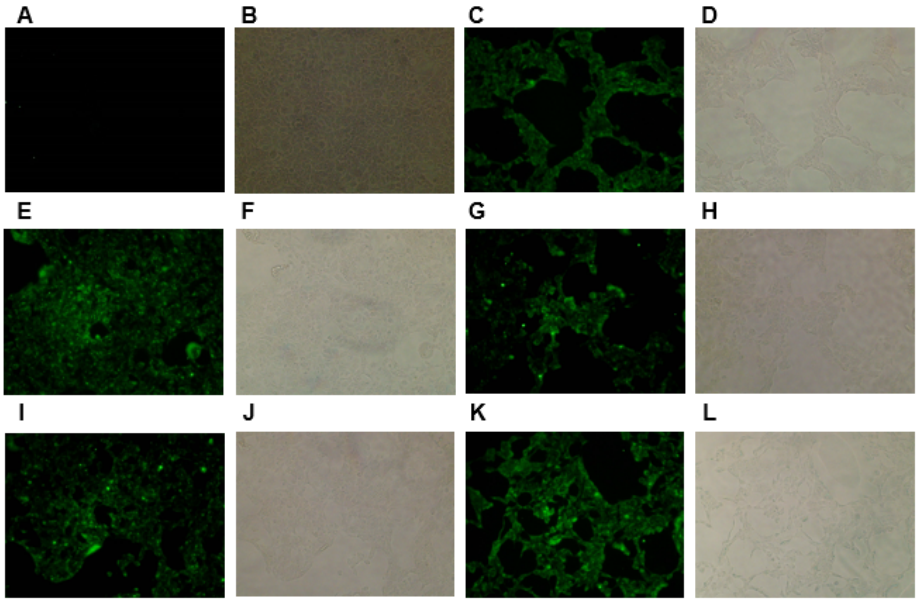

Figure 5.4. Fluorescence images of HEK293 cells expressing (A) no OR proteins, (C) OR2Z1 proteins, (E) OR4M1 proteins, (G) OR2A1 proteins, (I) OR5H1 proteins, and (K) OR5C1 proteins. Optical images of HEK293 cells expressing (B) no OR proteins, (D) OR2Z1 proteins, (F) OR4M1 proteins, (H) OR2A1 proteins, (J) OR5H1 proteins, (L) OR5C1 proteins. All images were taken at 100X amplification.

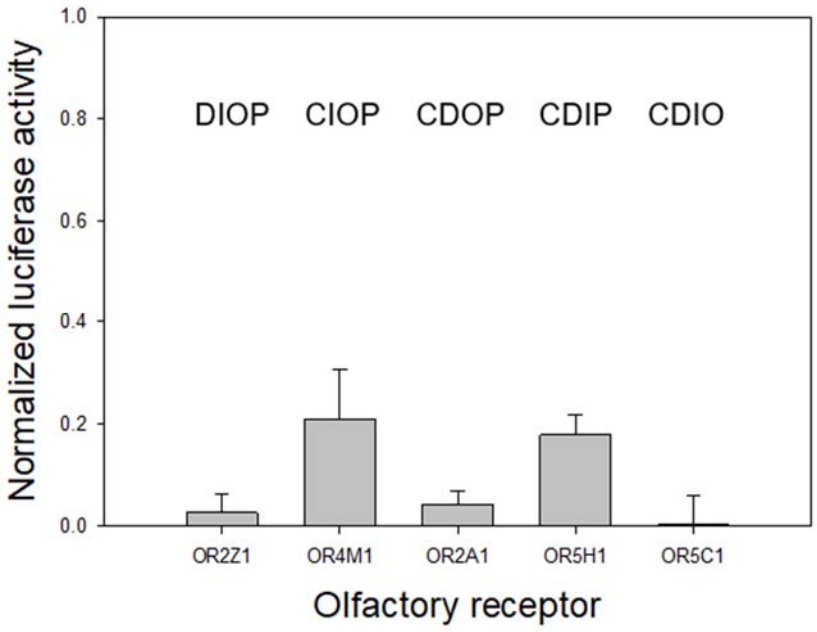

Figure 5.5. Selectivity test of screened hORs to 1 mM mixture of TB biomarkers excluding their ligand. Each alphabet means initial of chemicals (C: cymol, D: 2,6-dimethylstyrene, I: isopropyl acetate, O: O-xylene, P: 3-pentanol).

5.4 Conclusions

Bioelectronic sensor technology developed to detect VOCs more selectively requires a primary transducer capable of detecting biomarkers. The olfactory receptor protein possesses a function suitable for utilize as a primary transducer for sensing volatile organic compounds, but deorphanization process is necessary because the ligand of many olfactory receptors are still remain unknown. In this study, we screened the human olfactory receptor library to detect biomarkers of TB reported to be detected in urine, and identified 5 olfactory receptors that each detect 5 biomarkers. The membrane expression of OR proteins in HEK293 cells were confirmed by immunocytochemistry. Also, the responsivity and selectivity of each ORs to target biomarkers were also examined by dual-glo luciferase assay. Although 2 out of 5 ORs showed a slight lack of selectivity, the hORs we identified will be useful as primary transducers of bioelectronic sensors for the diagnosis of tuberculosis through further research in the future.

Chapter 6.

Identification and validation of gastric cancer biomarkers and assessment of human olfactory receptors for the biomarker detection

Chapter 6. Identification, validation of gastric cancer biomarkers and assessment of sensing function of human olfactory receptors for the biomarker detection

6.1 Introduction

Cancer is one of the leading causes of death worldwide, accounting for 16% of global mortality in 2016 [96]. In particular, gastric cancer accounts for 5.7% of the 18.1 million new cases of cancer seen each year and ranks third (8.2%) among cancer mortality [55]. The 5-year relative survival rate for patients diagnosed with stage IV gastric cancer in the United States was just 3.2 % [97]. Early diagnosis and treatment of gastric cancer is very important for the survival of patients. Currently, esophagogastroduodenoscopy, biopsy, culture test, and rapid urea test (RUT) are used as diagnostic techniques for gastric cancer. However, there is a problem in that gastric cancer is rarely diagnosed in most countries without regular health checkups. Because of the ambiguous, subjective symptoms of early gastric cancer, it is difficult for patients to decide to visit the hospital and receive an invasive and expensive diagnosis. Therefore, there is a need for a point-of-care diagnostic technology that is easy to employ in everyday life without visiting a hospital, similar to a blood glucose meter.

The development of new cancer diagnostic technologies relies on the search for biomarkers. Various kinds of chemicals, proteins, and peptides collected from the human body can serve as biomarkers. Recently, thanks to the development of technology such as gas chromatograph/mass spectrometry (GC/MS), researchers have found that the composition of volatile organic compounds (VOCs) in exhaled breath changes to reflect the condition of the body. So far, human secretions including blood [98, 99], saliva [100], urine [101], and feces [102] have been analyzed to find VOCs related to cancer. In addition, several studies have been conducted to characterize VOCs generated by tumor cell lines from headspace gas as cancer biomarkers [22, 74, 103]. One of the most promising sources of biomarkers is exhaled breath. The collection process of breath does not cause the subject any pain. Thus, many researchers have been working to find biomarkers of different types of cancer in exhaled breath [33, 60, 104].

The metabolic processes of cancer cells and normal cells are quite different from each other and the composition of metabolic products secreted by the

human body is likely to change if cancer is present [105]. Thus, previous studies on gastric cancer biomarkers that examined exhaled breath were conducted by comparing cancer patients with healthy controls and patients with benign diseases [25. 106]. However, factors such as the general condition of the patient, the varying chemical compositions of environmental gases in different regions, medications, and personal eating habits can affect the composition of exhaled breath. Therefore, it is necessary to confirm the influence of gastric cancer tumors in the body, excluding other effects as much as possible. Given this context, comparing exhaled breath samples taken from patients before and after surgery is very important to understanding the individual effects of tumor presence on components of exhaled breath, since the VOC profile of exhaled breath can also be altered by the general condition of the patient and the existence of inflammation in other bodily organs.

An oft-used tool for capturing gaseous VOCs is the solid-phase microextraction (SPME) fiber [107]. SPME allows for the capture of trace amounts of chemicals contained in mixed gaseous components or liquids without solvents [108]. Because of its ease of use and constant material improvements, SPME technology has been widely used to analyze drugs [109], food [110-112], flavor volatiles [113], water pollution [114], air pollution [115], cancer cell lines [116], urine [117], blood [118, 119], and breath [120-122]. SPME fibers are often used to adsorb VOCs in an effort to minimize the contamination or decomposition of a gas sample that may occur during transfer from the sampling place to the analytical instrument. Several volatile biomarker studies related to disease have also been conducted using SPME fibers [60, 123, 124]. Therefore, utilizing SPME fibers for collection of VOCs is considered an appropriate methodology for exhaled breath analysis.

In this study, we used SPME-GC/MS to analyze exhaled breath samples from gastric cancer patients taken at 3 time points, right before surgery, 1 week after surgery, and more than 1 month after surgery. Exhaled breath samples from healthy volunteers and samples from the surrounding air were also analyzed as negative controls. Moreover, we recruited not only advanced gastric cancer patients but also early gastric cancer patients to see if an identified breath biomarker could help in the early diagnosis of gastric cancer.

6.2 Selection of SPME fiber type

To choose an appropriate type of SPME fiber for collection of VOCs in breath, the exhaled breath of gastric cancer patients was collected with PDMS/DVB and CAR/PDMS fibers and analyzed by GCMS. These two types of SPME fibers have been widely used to collect various volatile chemicals in gases [125. 126]. As shown in Figure 6.1, the VOCs in exhaled breath were more efficiently collected using CAR/PDMS fibers. Therefore, all sampling procedures in this study were conducted with CAR/PDMS fibers.

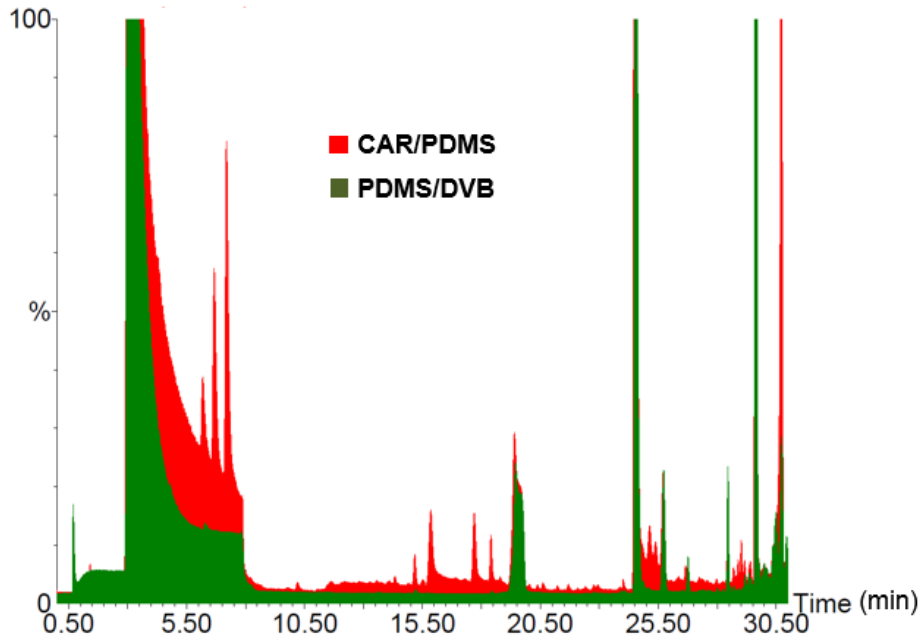

Figure 6.1. The chromatogram results of VOCs in the exhaled breath of a gastric cancer patient collected with CAR/PDMS fiber and PDMS/DVB fiber.

6.3 Sampling and analysis of exhaled breath

A total of 11 VOCs in the breath of gastric cancer patients and healthy subjects were identified using the SPME-GCMS method. The chromatographic results of exhaled breath analysis are shown in Figure 6.2, which reveals a different pattern for patients and healthy subjects. The retention time, m/z ratio, and median values of peak areas of the 11 VOCs in exhaled breath of gastric cancer patients and healthy subjects are listed in Table 6.2. We found that acetone, dichloromethane, hexane, 3-methylpentane, and toluene were more abundant in the exhaled breath of healthy controls than in that of gastric cancer patients. In contrast, the short-chain fatty acids (SCFAs) propionic acid, butyric acid, isovaleric acid, and 2-methylbutyric acid were more abundant in the exhaled breath of gastric cancer patients. The peak area of isobutyric acid was not significantly different between the two groups.

To distinguish between exogenous and endogenous VOCs in the exhaled breath, environmental gas was also collected from the place where breath sampling was conducted. The known exogenous VOC styrene [71] was excluded from the analytical target group in advance. The chromatogram results of exhaled breath from gastric cancer patients and environmental gas are compared in Figure 6.3. As shown in Figure 6.4, toluene was found to be very abundant in environmental gas. Also, the amount of isobutyric acid was relatively higher in the environmental gas than in the exhaled breath.

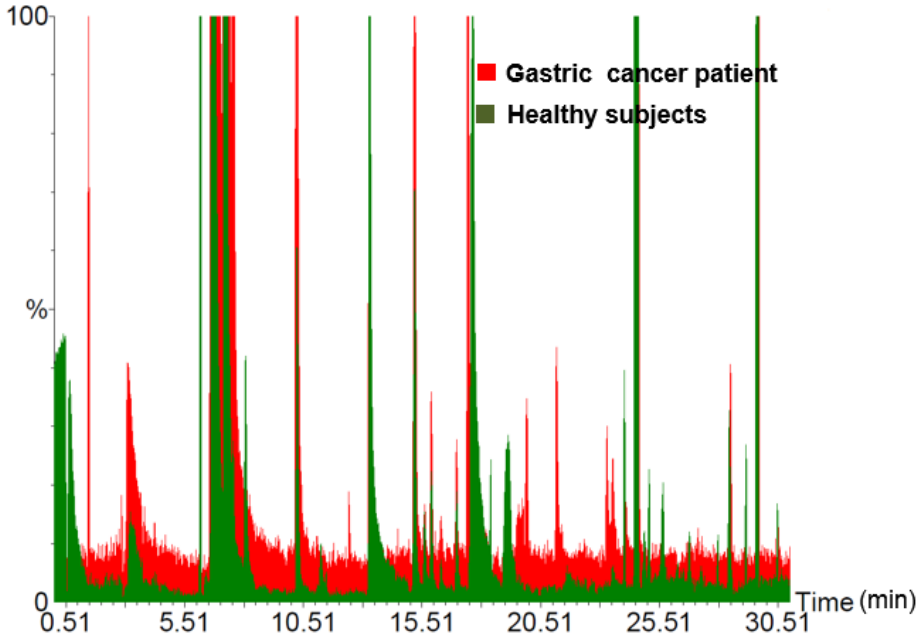

Figure 6.2. Comparison of VOC profiles in the exhaled breath of gastric cancer patient and healthy subject.

Table. 6.1. Median values of peak areas of VOCs in exhaled breath of gastric cancer patients and healthy subjects

Compound	Retention time (min)	m/z	Median values of peak areas[a] (interquartile range)		p^b
			Gastric cancer patients ($N = 64$)	Healthy subjects ($N = 61$)	
Isoprene	6.60	67	7132 (1066 – 13578)	9013 (6626 – 14306)	0.00228
Acetone	7.14	43	7819 (1454 – 16320)	16457 (10300 – 30591)	<0.0001
Dichloromethane	8.22	49	2 (0 – 112)	33983 (7994 – 89641)	<0.0001
3-Methylpentane	8.95	57	6 (0 – 33)	41 (29 – 74)	<0.0001
Hexane	9.15	57	1 (0 – 17)	471 (244 – 1022)	<0.0001
Propionic acid	17.14	74	191 (2 – 533)	0 (0 – 1)	<0.0001
Toluene	18.40	91	2894 (713 – 6097)	14711 (10537 – 19680)	<0.0001
Isobutyric acid	19.90	43	3 (0 – 67)	5 (2 – 28)	0.6284
Butyric acid	21.18	60	23 (1 – 298)	1 (0 – 1)	<0.0001
Isovaleric acid	22.88	60	2 (0 – 38)	0 (0 – 1)	0.0066
2-Methylbutyric acid	23.17	74	1 (0 – 65)	0 (0 – 1)	0.0286

[a]Raw value x 10^{-3}, [b]Mann-Whitney test.

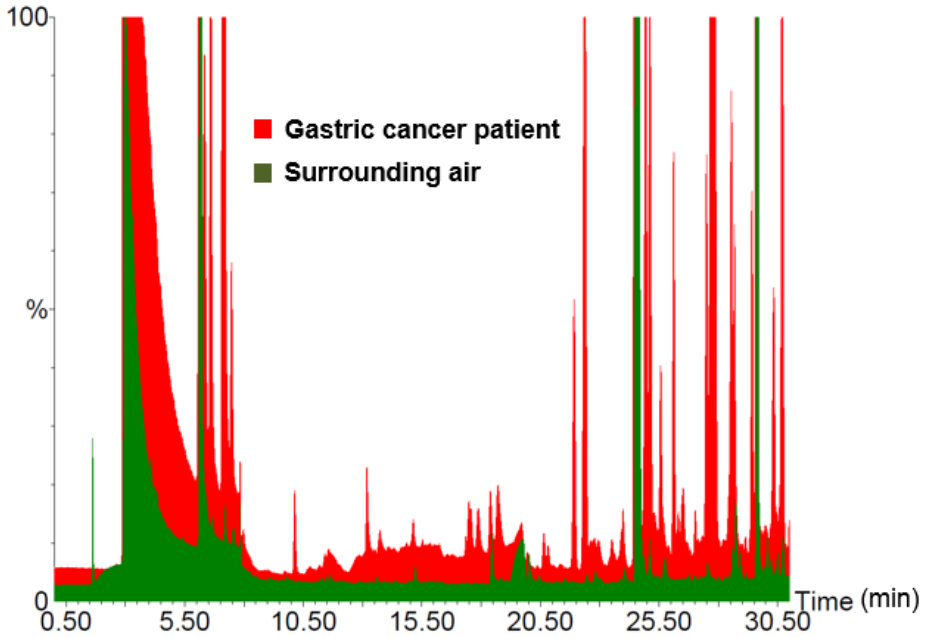

Figure 6.3. The chromatogram results of VOCs in the exhaled breath of a gastric cancer patient and environmental gas.

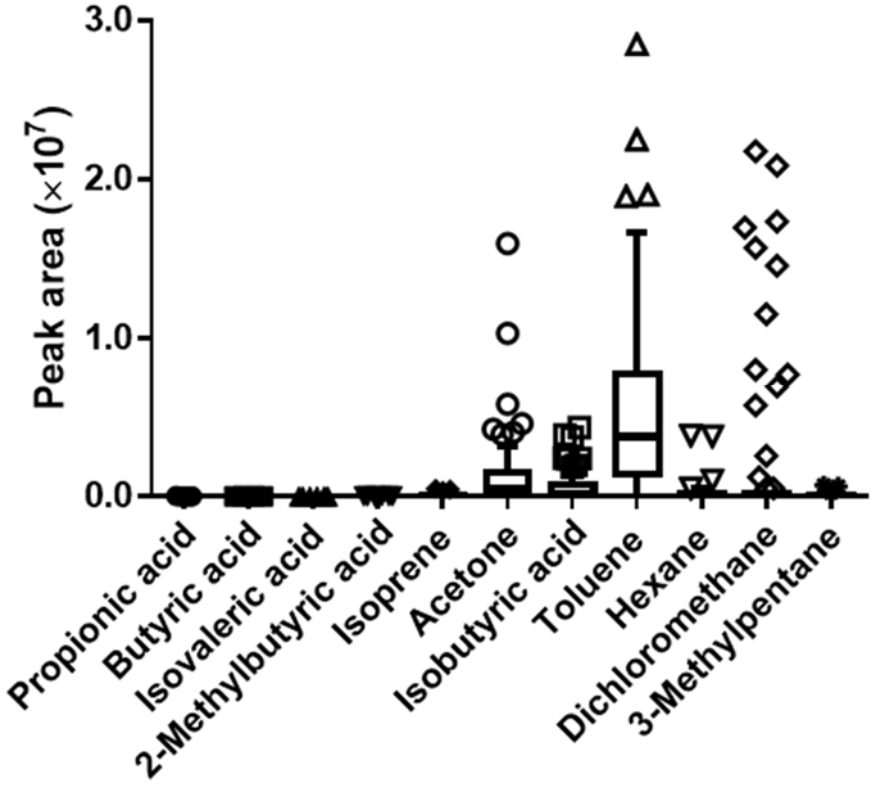

Figure 6.4. Peak area of VOCs in environmental gas. The points above a box plot are outliers which were calculated with Tukey's method.

6.4 Changes in the amounts of VOCs in the breath of gastric cancer patients before and after surgery

The chromatogram results of exhaled breath from gastric cancer patients before and after surgery is compared in Figure 6.5. The amounts of some VOCs in exhaled breath of gastric cancer patients whose tumors were removed from the body by surgery showed a significant change compared to the preoperative results (Figure 6.6). In particular, the levels of butyric acid and propionic acid in exhaled breath were markedly decreased after tumor resection. These two VOCs tend to be further reduced in the exhaled breath of patients more than one month after surgery, suggesting a direct correlation with the presence of gastric cancer cells inside a patient's body. The amount of isovaleric acid and 2-methylbutyric acid in exhaled breath were also decreased after surgery, but the level of change was found to be insignificant.

Previous studies have reported that SCFAs have a strong relationship with gastrointestinal cancer. Some SCFAs, including acetic acid, butyric acid, and propionic acid, are known to be produced by anaerobic fermentation of dietary carbohydrates by colonic bacteria [127, 128]. Butyric acid and propionic acid are also known to induce apoptosis of a gastric carcinoma cell line [129]. In addition, sodium butyrate has been reported to be associated with apoptosis of colon cancer cells [130, 131]. Therefore, monitoring of SCFAs is likely to be important for understanding the activity of cancer cells in the digestive tract. Moreover, some reports have claimed that SCFAs serve as potent biomarkers in the exhaled breath of gastric cancer patients. Butyric acid [132], hexanoic acid [133], and pentanoic acid [134] in particular have been verified through cross-platform validation, suggesting that they are likely to be biomarkers of gastrointestinal cancer in the breath.

The concentration of acetone was noticeably increased after surgery, but decreased again as the patients stabilized. This result supports the hypothesis that the amount of acetone in breath reflects the effects of stress on the human body. For example, researchers have reported that the amount of acetone in the breath varies with oxidative and metabolic stress during cardiac surgery [135]. The concentration of acetone in breath is known to reflect various changes in the body. The concentration of acetone in breath increases after exercise [136] and fasting [137], which is related to blood glucose levels. Acetone in exhaled breath is also considered to be a potent biomarker of diabetes mellitus [138].

However, no close link was found between gastric cancer and acetone in breath during this study.

Diagnosing gastric cancer by detecting specific VOCs in exhaled breath is noninvasive and simple, but it would be most useful if cancer could be diagnosed at an early stage when the subjective symptoms of a patient are not clear. Thus, we separately identified patients in early stages of gastric cancer to track the quantitative changes of VOCs in exhaled breath. Notably, butyric acid and propionic acid, which were selected as the putative biomarkers for gastric cancer in this study, showed significant quantitative differences in the exhaled breath of early gastric cancer patients vs. healthy controls (Figure 6.7). Although isovaleric acid, 2-methylbutyric acid, and isobutyric acid belong to the same SCFA category, there was no significant difference in the amount of these VOCs in the exhaled breath of patients with early gastric cancer and healthy subjects.

Since only 18 patients with early-stage gastric cancer participated in this study, it is difficult to draw meaningful conclusions based on these results alone. However, assuming that the development of cancer cells is proportional to the emission of specific VOCs, these results can be considered to increase the reliability of the two newly-discovered biomarkers.

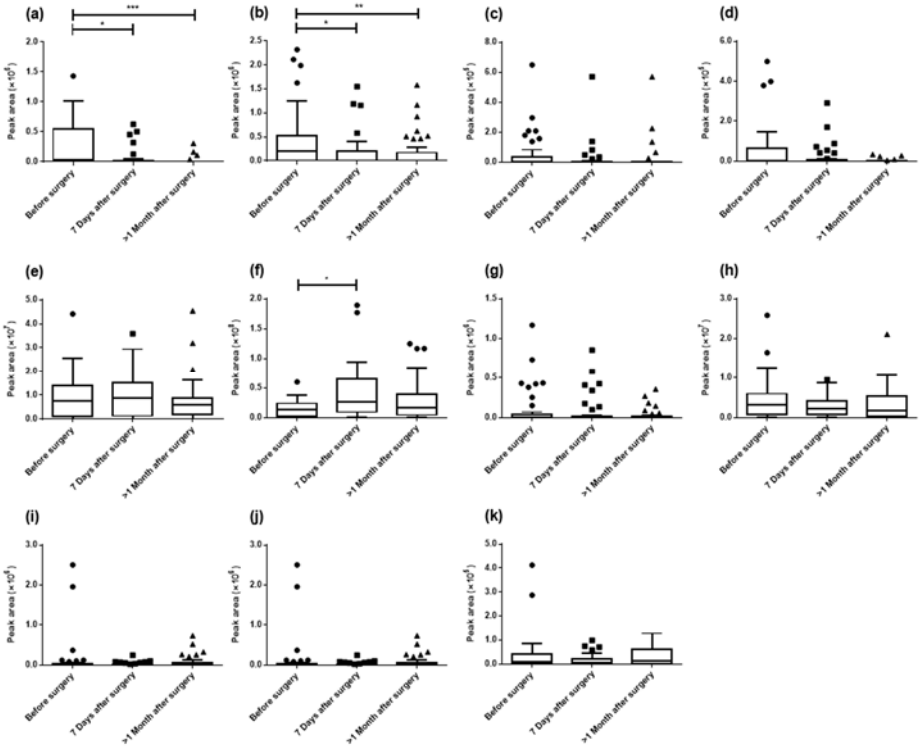

Figure 6.5. Quantitative change of (a) butyric acid, (b) propionic acid, (c) isovaleric acid, (d) 2-methylbutyric acid, (e) isoprene, (f) acetone, (g) isobutyric acid, (h) toluene, (i) hexane, (j) dichloromethane and (k) 3-methylpentane in the exhaled breath of gastric cancer patients before and after surgery. A non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (significance of results, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$). The points above a box plot are outliers which were calculated with Tukey's method.

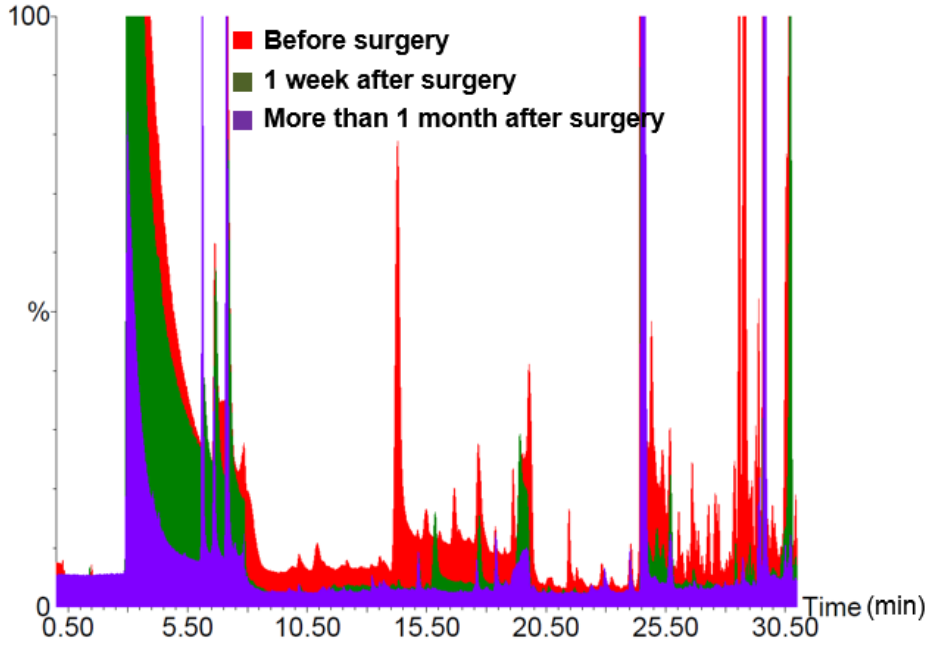

Figure 6.6. Comparison of 3 chromatogram results of the exhaled breath collected from a gastric cancer patient before and after surgery.

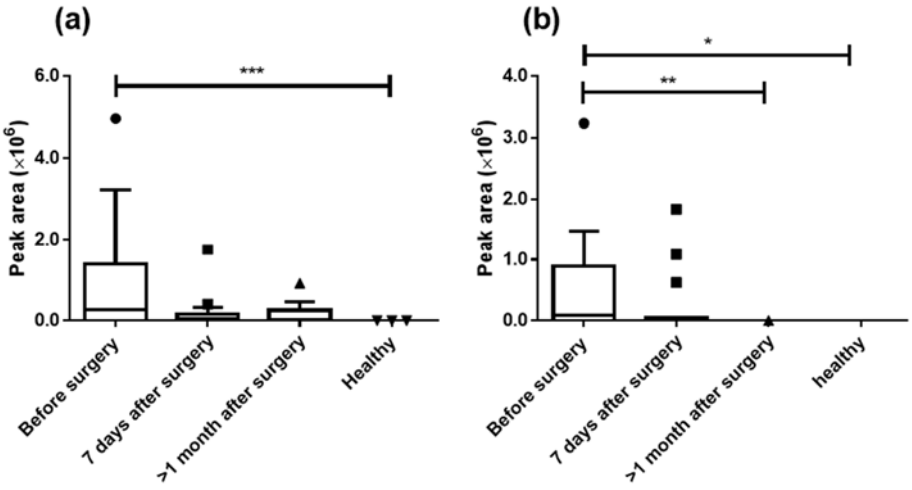

Figure 6.7. Quantitative change of (a) propionic acid and (b) butyric acid in exhaled breath of early gastric cancer patients (stage 1). A non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (significance of results, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$). The points above a box plot are outliers which were calculated with Tukey's method.

6.5 Statistical analysis for construction of diagnostic model

In order to distinguish gastric cancer patients from healthy subjects, statistical analysis was performed based on the quantitative changes in VOCs that are likely to be related to the presence of gastric cancer tumors in the body. Initially, we conducted a statistical analysis that included not only quantitative changes in VOC levels but also information on the subjects including sex, health status, age, and any complications except cancer. But clinical evidence for these factors on cancer development was difficult to find, so all elements except for the level of VOCs were excluded from the final statistical model. Considering the multicollinearity of the analysis data, the Lasso regression method was adopted as the model prediction method. The outcome variable was gastric cancer (patient group: 1, control group: 0), and four SCFAs (butyric acid, propionic acid, isovaleric acid, 2-methylbutyric acid) were considered as independent variables. At this time, the unit of each VOC for odd ratio (OR) estimation was obtained by dividing the peak area value by 100,000. As a result, butyric acid and propionic acid were included in the final model for gastric cancer diagnosis (Table 6.3). The AUC for the ROC curves used to discriminate gastric cancer patients from healthy subjects was 0.829 (Figure 6.8). The c-index on the basis of the AUC for predicted probability is 0.3340. The cut-off value was identified on the basis of the highest Youden's index. The sensitivity and specificity in predicting gastric cancer (cut-off value > 0.3340) are 72.13% (95% confidence interval of 59.2 to 82.9) and 95.08% (95% confidence interval of 86.3 to 99.0), respectively.

In addition, in order to validate the present diagnostic model, an additional 10 gastric cancer patients and 10 healthy controls were recruited. Breath samples were collected in compliance with the above protocol, and then the subjects were diagnosed using our statistical model. As shown in Figure 6.9, the diagnosis was conducted by calculating the equation in Table 6.3-(a). In this confirmatory analysis, the sensitivity and specificity of the present diagnostic model were found to be 100% and 90%, respectively. Although the size of the additional study group is not sufficient to be statistically significant, the diagnostic model is considered to be worth further development through additional validation studies.

Table 6.2. Construction of the diagnostic model with the LASSO regression method

(a) Parameter of estimates

	Degree of freedom	Estimate	Odd ratio
Butyric acid	1	0.6153	1.8502
Propionic acid	1	0.4837	1.6237

ln(p/(1-p)) = -0.7193 + 0.6153 * (peak area of butyric acid / 100000) + 0.4847 * (peak area of propionic acid / 100000)

(b) The diagnostic power of predicted probability for gastric cancer

Model	Area under ROC curve	Probability	Sensitivity	Specificity
LASSO regression	0.829	> 0.3340	72.13	95.08

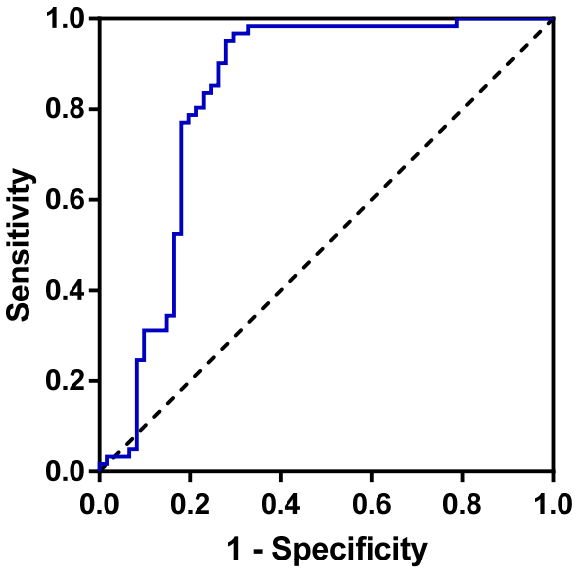

Figure 6.8. ROC curve of the diagnostic model for the discrimination of gastric cancer patients from healthy subjects using the peak areas of butyric acid and propionic acid. The AUC was 0.829 (95% confidence interval of 0.7476 to 0.9111, $p < 0.001$).

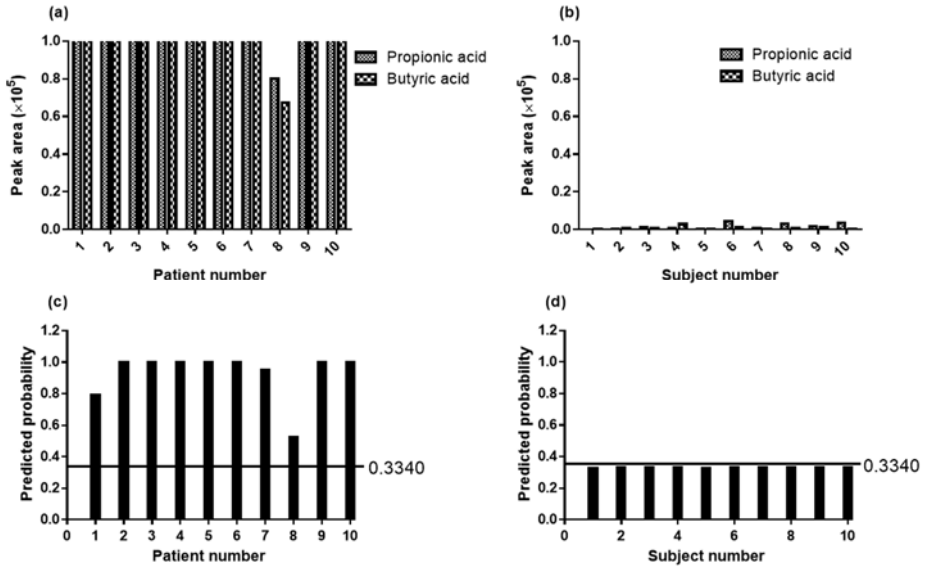

Figure 6.9. Validation of the present diagnostic model. Peak areas of propionic acid and butyric acid in the exhaled breath of (a) 10 additional gastric cancer patients and (b) 10 additional healthy subjects were analyzed by SPME-GC/MS. The prediction probability values of (c) gastric cancer patients and (d) healthy volunteers were obtained by applying the diagnostic model in table 6.3-(a).

6.6 Cell-based assay for characterization of human olfactory receptors recognizing gastric cancer biomarkers

Appropriate sensors are needed to distinguish gastric cancer patients by detecting biomarkers in the exhaled breath. Sensors for detecting the volatile biomarker contained in the breath have been developed on various platforms, and among them, we decided that the bioelectronic sensor is suitable for selective and sensitive detection of VOCs. Therefore, biological sensing molecules to be used as primary transducers are required, and researchers have successfully introduced the human olfctory receptors to bioelectronic sensors recently [139, 140]. It was previously reported that human olfactory receptor OR51E1 and OR51E2 can detect butyric acid and propionic acid, respectively [141, 142]. We used the dual-glo luciferase reporter gene assay method in HEK293 cells to confirm the responsivity to butyric acid and propionic acid when transiently expressing OR51E1 and OR51E2. As shown in Figure 6.10, it was confirmed that OR51E1 and OR51E2 were able to detect butyric acid and propionic acid, respectively. In addition, a selectivity test was conducted to confirm whether olfactory receptors and gastric cancer biomarkers cross-react with each other (Figure 6.11). As a result, it was confirmed that the responsivity of OR51E1 to butyric acid was significantly higher than that of propionic acid, and OR51E2 selectively recognized propionic acid. However, since OR51E2 is known to detect not only propionic acid but also acetic acid, additional preliminary investigation will be required to use these olfactory receptors as a sensing material for bioelectronics sensor detecting the exhaled breath in which multiple VOCs are mixed.

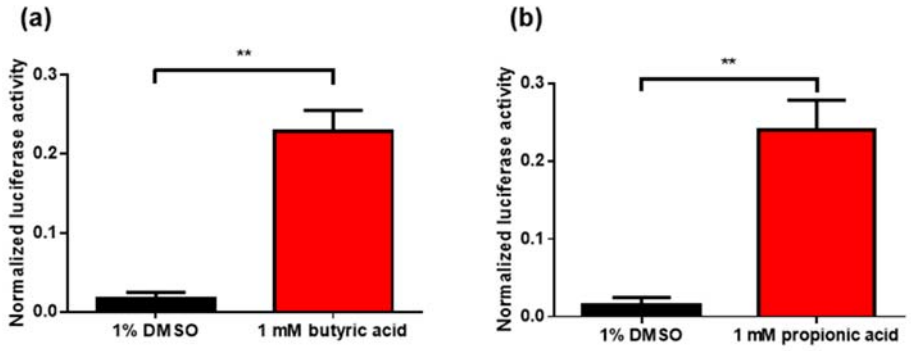

Figure 6.10. Responsivity of (a) OR51E1 to butyric acid and (b) OR51E2 to propionic acid. Butyric acid and propionic acid were diluted in dPBS containing 1% DMSO at a concentration of 1 mM. A non-parametric comparison of two group was conducted with Mann-Whitney test (significance of results, ** : $p < 0.01$, n=8~12).

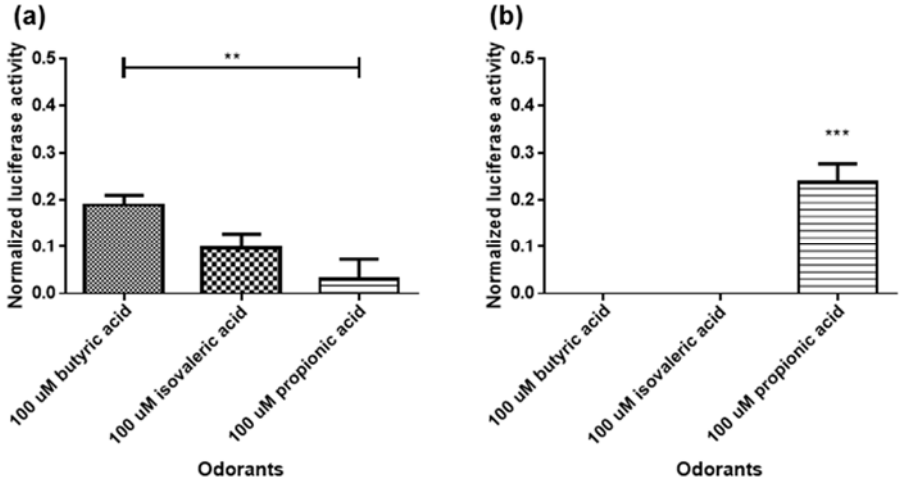

Figure 6.11. Selectivity of (a) OR51E1 to butyric acid and (b) OR51E2 to propionic acid. A non-parametric multiple comparison of each group was conducted with Kruskal-Wallis and Dunn's test (significance of results, ** : $p < 0.01$, *** : $p < 0.001$).

6.7 Conclusions

This study focused on finding more tumor-specific VOCs than had been identified in previous breath biomarker studies. To this end, bodily condition as reflected in the exhaled breath of gastric cancer patients was analyzed three times in a relatively short period of time before and after a tumor resection. Eleven VOCs were identified in the exhaled breath of gastric cancer patients and healthy subjects. Of these molecules, 2 SCFAs, butyric acid and propionic acid, decreased in abundance with the removal of gastric cancer tumors. The median peak areas of these SCFAs were significantly lower in the exhaled breath of the healthy subjects than in that of the preoperative gastric cancer patients. Also, a statistical model was constructed to distinguish patients from healthy subjects based on the peak areas of the two SCFAs. In addition, we conducted a cell-based luciferase reporter gene assay for confirmation of detection performance of putative human olfactory receptors recognizing butyric acid and propionic acid. It was confirmed that OR51E1 and OR51E2 can sensitively and selectively detect each SCFAs. We expect to use these human olfactory receptors to develop bioelectronic sensors for gastric cancer diagnosis.

This study is about not only screening for gastric cancer biomarkers in the breath, but also building a diagnostic model that can be used to distinguish cancer patients from healthy subjects by utilizing a quantitative value of target VOCs. Certainly, further research is needed to validate our results. But we believe that the present study will advance the noninvasive diagnostic methods of gastric cancer by identifying SCFAs as biomarkers of early-stage gastric cancer.

Chapter 7.

Overall discussion and further suggestions

Chapter 7. Overall discussion and further suggestions

Volatile organic compounds (VOCs) are defined as all organic substances with volatility that occur naturally or artificially. In particular, branch of chemistry that is used to diseases diagnosis or health monitoring by studying VOCs from a biological system under a specific environment is called volatolomics. Sensor technology for detecting such disease-related biomarkers has also been developed. In particular, bioelectronic sensors show high selectivity and sensitivity by fusion of nano-structured semiconductors and bio-derived sensing materials. Olfactory receptor is an example of a biological molecule that is commonly used as a primary transducer of a bioelectronic sensor, and it is expected that the range of application of bioelectronic sensors including olfactory receptors will be as wide as humans can distinguish more than 1 trillion odor combinations [39].

In the thesis, headspace gas of cancer cell lines and breath of gastric cancer patients were analyzed for identification of specific biomarkers. And, the human olfactory receptors were screened for detection of disease biomarkers.

In chapter 4, a lung cancer-specific biomarker was identified, and screening of human olfactory receptors capable of detecting lung cancer biomarkers was conducted. Lung cancer cell line SK-MES and normal lung cell line MRC-5 were cultured, and VOCs in headspace gas were collected and analyzed using SPME-GC/MS. 2-Ethyl-1-hexanol was selected as a potential biomarker for lung cancer. The human olfactory receptor capable of detecting 2-ethyl-1-hexanol was screened, and its responsivity and selectivity were measured. In addition, olfactory nanovesicles, an artificial olfactory cell, were produced for further use as a primary transducer for bioelectronic sensors. It was confirmed that these olfactory nanovesicles still possess the ability to detect 2-ethyl-1-hexanol as olfactory receptor proteins were expressed in cells. This approach would be useful for effectively diagnosis of diseases that the biomarkers have not yet been identified.

In chapter 5, human olfactory receptors that detect 5 tuberculosis biomarkers have been screened. Responsivity of olfactory receptors to each biomarker was measured, and selectivity to a mixture biomarkers was also measured. These results provide a method for determining which sensory receptor would be a good candidate for a biological sensing element when it is necessary to detect mixed biomarkers.

In chapter 6, gastric cancer biomarkers found in exhaled breath were identified. The exhaled breath of gastric cancer patients and healthy subjects were collected and analyzed by using SPME-GC/MS. 2 Short-chain fatty acids (SCFAs) were selected as candidates for gastric cancer biomarkers, and through statistical analysis, a diagnostic model was constructed to distinguish patients with gastric cancer using the amount of biomarkers in the exhaled breath. The sensitivity and specificity in diagnosing gastric cancer by this model were 72.13% and 95.08%, respectively. For validation of constructed diagnostic model, breath samples of additional 10 gastric cancer patients and 10 healthy subjects were collected and analyzed, and sensitivity and specificity were estimated to be 100% and 90%, respectively. In addition, the responsivity and selectivity of human olfactory receptors reported to detect multiple SCFAs, including two gastric cancer biomarker candidates, were measured.

SPME-GC/MS is the primary method used to find disease-specific biomarkers in this study. SPME is an effective instrument for collecting various VOCs, and the type of VOCs that are effectively collected differs depending on the composition of the porous polymer fiber. Among them, CAR/PDMS fibers are specialized for collecting gaseous VOCs, and VOCs in the headspace gas of water [143], human fecal VOCs [144], microbial VOCs [145] and VOCs emitted from polymers [146] have been collected by CAR/PDMS fiber. PDMS/DVB fiber has also been used to capture gaseous VOCs [147, 148], it was found in this study that the efficiency of capturing VOCs present in human exhaled breath is lower than that of CAR/PDMS fiber.

GC / MS is the most widely used equipment for gas component analysis, and optimization of its operating protocol is a key process for screening of target chemicals. In this study, chromatographic protocols were established to effectively identify disease-related VOCs in headspace gas of cells and exhaled breath with reference to method of other biomarker studies. However, since the efficiency of chromatographic protocol differs greatly depending on the type of column, sample, and instrument of collection, it is necessary to optimize each study depending on the experimental condition.

Lung cancer is the most commonly diagnosed cancer worldwide [55]. Since the lesion of lung cancer is directly connected to the respiratory tract, many biomarker studies focused on exhaled breath have already been conducted. However, since VOCs in the esophagus and oral cavity are mixed with VOCs generated by cancer cells in exhaled breath, there is a possibility that it is a complexed sample to identify specific VOCs produced by cancer cells

themselves. Therefore, we identified the biomarker of lung cancer called 2-ethyl-1-hexanol by directly analyzing the headspace of lung cancer cell lines and normal lung cell lines. In addition, non-specific VOCs could be excluded from the biomarker candidate by additionally analyzing and comparing the headspace gas of the culture medium. Because most lung cancer biomarker studies have been conducted in different ways for different type of cells or patients, the results are somewhat equivocal and the number of common biomarkers was very small. However, we found that 2-ethyl-1-hexanol is a more reliable biomarker of lung cancer because the possible production process in the human body has been confirmed and 2-ethyl-1-hexanol has also been reported as a lung cancer biomarker in studies using other VOC sources and analytical equipments. Cross-validation with the VOCs reported in the previous exhaled breath biomarker study [71] led to the conclusion that 2-ethyl-1-hexanol is a highly potent lung cancer biomarker.

Tuberculosis is a highly contagious disease caused by infection with *Mycobacterium* tuberculosis. Since *Mycobacterium* tuberculosis can invade not only the lungs but also various organs, the source candidate for tuberculosis-specific biomarkers is diverse. In this study, we screened olfactory receptors that detect 5 biomarkers [95] specified in urine, a non-invasive biomarker source. In order to diagnose a disease based on multiple biomarkers in a mixed state, selectivity of a sensing element becomes important. Therefore, we have confirmed that each olfactory receptor possesses selectivity for a ligand against several biomarker mixtures, not just to select five olfactory receptors capable of detecting biomarkers.

The 5-year relative survival rate for stage IV gastric cancer patients is extremely low [97]. Therefore, early diagnosis of gastric cancer is very essential for survival of patients. However, because the initial symptoms of gastric cancer are unclear, it is not easy for patients to visit the hospital for a precise inspection such as a esophagogastroduodenoscopy. So gastric cancer is a disease that urgently demands the development of a non-invasive and simple diagnostic technology, and the exhaled breath test satisfies this requirement. Control of the specimen is essential because exhaled breath is a mixture of VOCs from various human organs as well as cancer cells. To reduce this problem, in this study, we asked the volunteer's mouth to be washed and overnight fasted before collection of exhaled breath. Breath samples from 64 gastric cancer patients and 61 healthy subjects were collected and analyzed. It was confirmed that butyric acid and propionic acid were present in a relatively

large amount in the gastric cancer patient's breath. Through statistical analysis, a diagnostic model using two SCFAs as a biomarker was developed. And additionally collected breath samples of gastric cancer patients and healthy subjects were also successfully distinguished by this diagnostic model. This result is in line with recent studies that estimate SCFAs as putative biomarkers for gastric cancer [133, 134]. Conventional disease biomarker studies are carried out in only one of three factors, including biomarker discovery, construction of diagnostic model, and validation study. The reason is that there are manpower issues required for research, but it also requires research capabilities in various fields such as chemical engineering, biotechnology, medicine, and statistics step by step in the research process. We have recruited volunteers under strict rules, collected samples under controlled conditions, and analyzed a larger number of samples compared to other cancer-related biomarker studies for construction of diagnostic model. Also, although not a large number, we have seen the possibility that this diagnostic model will work correctly for patients outside of the study group. In this respect, we believe that we have found a reliable biomarker of gastric cancer based on consistent and abundant research data compared to previous disease biomarker studies.

To sum up this thesis, biomarkers for various diseases were identified by using SPME-GC/MS. Moreover, human olfactory receptors suitable for the role of sensing elements capable of detecting biomarkers of diseases have been screened. In table 7.1, newly identified disease biomarkers and olfactory receptors screened for biomarker detection in this thesis are summarized. This study suggests a methodology for discovering biomarkers for non-invasive and simple disease diagnosis. The biomarkers identified in this thesis may require additional validation through further studies, but it is encouraging that they have some continuity with previously reported studies. Biomarker research on human-derived VOCs will further expand the scope of application not only for disease diagnosis but also for health monitoring, and studies on sensing elements for biomarker detection will be further diversified. We hope that the proposed set of research achievements will help advance disease diagnosis technology.

Table 7.1. Disease biomarkers and olfactory receptors.

Disease	Biomarker	Source	Human olfactory receptor	Reference
Lung cancer	2-Ethyl-1-hexanol	Breath, **cancer cells***	**hOR4D11P***	[1]
	Heptanal	Blood	hOR1J2	[2]
	Hexanal	Blood, breath	hOR2W1	[2], [3]
Tuberculosis	O-xylene	Urine	**hOR5H1***	[4]
	Isopropyl acetate	Urine	**hOR2A1***	[4]
	Cymol	Urine	**hOR2Z1***	[4]
	3-Pentanol	Urine	**hOR5C1***	[4]
	2,6-Dimethylstyrene	Urine	**hOR4M1***	[4]
Gastric cancer	Butyric acid	**Breath***	hOR51E1	[5]
	Hexanoic acid	Breath	hOR52D1	[6]
	Pentanoic acid	Breath	hOR52D1	[5]
	Propionic acid	**Breath***	hOR51E2	
Colorectal cancer	Butyric acid	Feces	hOR51E1	[7]
	Isovaleric acid	Feces	hOR52D1	[7]
	Acetic acid	Feces	hOR51E2	[7]
	Nonanal	Breath	hOR1G1	[8]
	Decanal	Breath	hOR1G1	[8]

***Identified or screened in this study**

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Appendix 1.

Comparative evaluation of sensitivity to hexanal between human and canine olfactory receptors

Appendix 1. Comparative evaluation of sensitivity to hexanal between human and canine olfactory receptors

A1.1 Abstract

It is known that a dog possesses much better sense of smell than a human. It has been reported that a dog has advantages (compared to a human) in the number of olfactory receptor (OR) genes, proteins and cells, and also nasal structure. However, a definitive reason for superior sensitivity of the canine olfactory system remains controversial. In this study, we compared sensitivity of human and canine olfactory receptors relative to the same condition. Human OR (hOR2W1) and canine OR (cfOR0312), previously identified to recognize hexanal, were inserted to the pcDNA3 vector. This vector was transfected to HEK293 cells. Hexanal-discriminating ability of ORs was confirmed using Fura-2 AM, a dye which illuminates when binding to calcium ions flowing into cells once olfactory signaling occurs. Consequently, cfOR0312 was more sensitive to hexanal than hOR2W1. Comparison of OR sensitivity would be one of the major factors in clarifying difference of sensitivity between human and canine olfactory systems.

A1.2 Introduction

Sense of smell is an essential function in the survival and reproduction of species. However, sense of smell has only just begun to be understood. The progress of gene cloning and transfection technologies allows scientists to study the function and mechanism of odor sensing. The mammalian olfactory system remained largely unknown before the study of Axel and Buck [1]. Olfactory receptor genes of various organisms, of humans as well as other animals such as dogs [2], insects [3-6], and fish [7-9] have been studied, and research on the difference in olfactory function between human and other animals has also been initiated.

It has been considered that a dog has much better sense of smell than a human [10, 11]. Many researchers have reported reasons why a dog possesses superior olfactory sense than a human. For example, it has been reported that the number of functional olfactory receptor genes versus pseudogenes [12, 13], structure of the nasal cavity [14], density of neuronal cells and number of ORs

on the surface of olfactory epithelium [15] may affect olfactory sensitivity. Conversely, the claim about inferiority of human sense of smell, compared to rodents and dogs, has been met with counter arguments [16]. It has been reported that humans were more sensitive than mice or monkeys to 3-mercapto-3-methylbutyl-formate [17]. Similarly, human subjects were more sensitive than mice to mammalian blood odor component trans-4,5-epoxy-(E)-2-decanal [18]. Also, there is a body of evidence that shows, human behavior is strongly influenced by olfaction. Odors can influence perceived stress [19] and the peripheral nervous system [20] in humans. Consequently, the claim that the olfactory function of a human has deteriorated because it becomes less important in the evolutionary process has been strongly disputed.

Herein, we present sensitivities of the ORs as factors affecting the difference in olfactory abilities. To compare olfactory sensitivity of different species, sensory evaluation as well as assessment at the molecular level, may be effective. However, molecular level approaches to the ORs have not progressed sufficiently. For example, deorphanization of ORs has been conducted by many research groups with intracellular calcium ion quantification [21], luciferase assay [22], and cAMP assay [23]. But ligands of most human ORs have not been identified. So, it was necessary to select an identified ligand for sensitivity comparison at the molecular level. We chose hexanal as a target ligand. Because hexanal is an indicator of lipid oxidation, this chemical has been studied extensively as a spoilage marker of food, and a lung cancer biomarker in blood [24]. The ORs detecting hexanal have been reported. And hOR2W1, the most sensitive receptor to hexanal in human OR gene repertoire [25], was selected to represent sensitivity of a human to hexanal. Also, cfOR0312 was selected as a representative canine OR, because cfOR0312 has been reported as one of the most sensitive receptors in the canine OR gene repertoire [26]. Moreover, these receptors can be used to fabricate bioelectronic sensors to assess food quality by detecting hexanal [27, 28]. It is remarkable that studies such as incorporating several g-protein coupled receptors (GPCRs) [29, 30] and peptides [31, 32] to chemical sensors have been conducted because of excellent sensitivity and selectivity of biotropic receptors.

Previous studies on ORs at the molecular level have been conducted under different conditions, – host cell, expression vector, transfection reagent – so it was difficult to fairly compare performance of the ORs. Thus, we prepared the same experimental condition to evaluate sensitivity of human and canine ORs. By cloning and expressing these ORs to mammalian cells, we compared

sensitivity of human and canine ORs to hexanal. This study is the first to compare human and dog ORs in the same experimental environment at the molecular level.

A1.3 Cloning of hOR2W1 and cfOR0312 genes

The coding sequence of hOR2W1 and cfOR0312 was amplified by PCR using primers containing EcoRI and XhoI sites, and confirmed by electrophoresis (Figure A1.1). The size of intact OR genes was approximately 1 kb. These OR genes were inserted to pcDNA3 vector, for mammalian expression. For membrane targeting of OR proteins in HEK293 cells, Rho-tag sequence was inserted to N-terminus of both OR genes using HindIII sites. The Rho-tag sequence, was known to induce surface expression of membrane proteins in eukaryotic cells [33].

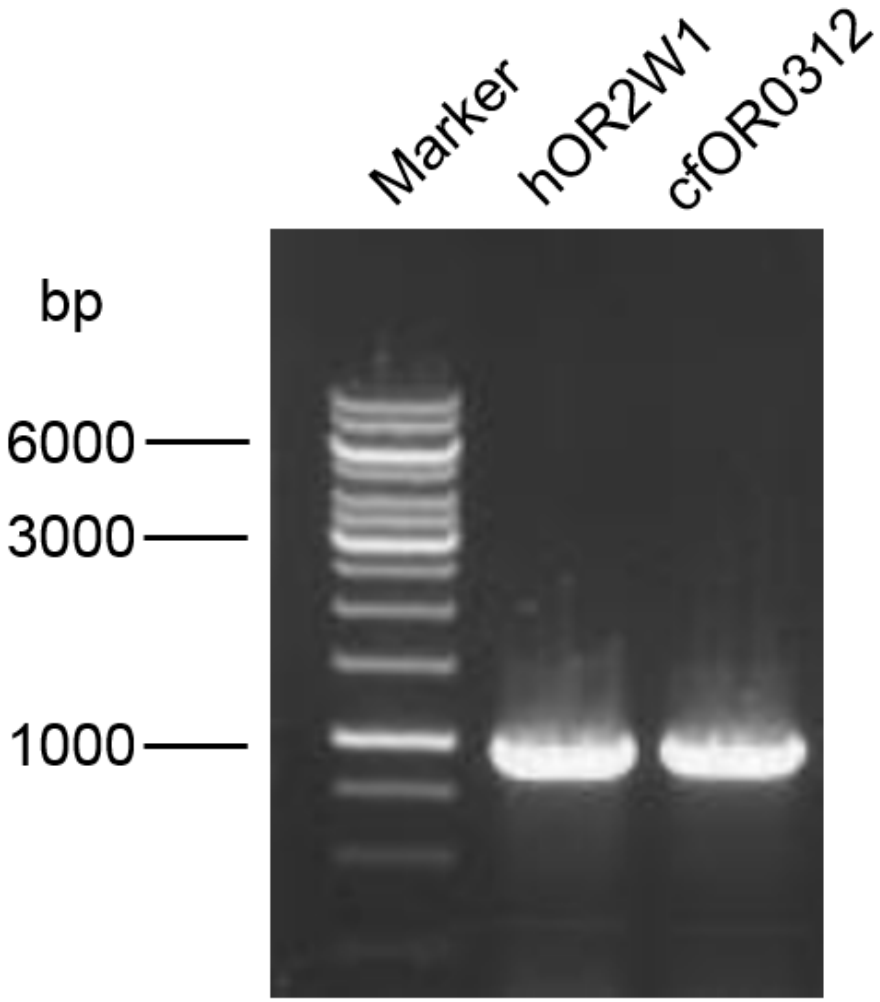

Figure A1.1. Gel electrophoresis image of hOR2W1 and cfOR0312 genes.

A1.4 Expression of ORs in HEK293 cells

HEK293 cells were transfected with pcDNA3 vectors containing hOR2W1 and cfOR0312 genes. After transfection, HEK293 cells were incubated in 96 well cell culture plates, for 24 h for adhesion and growth. Five times of PBS washing were conducted, after each step in the immunocytochemistry procedure, such as fixation, primary antibody solution, and fluorescent dye-conjugated secondary antibody solution treatment. Membrane expression of OR proteins on HEK293 cells was detected by immunocytochemistry, using anti-Rho tag antibody (Figure A1.2A and A1.2C). Optical images of fixed cells were taken, for identification of cell confluency (Figure A.2B and A1.2D). The expression rate of each ORs were quantitatively compared (Figure A1.2E) by using the corrected total cell fluorescence (CTCF) with ImageJ software [34, 35]

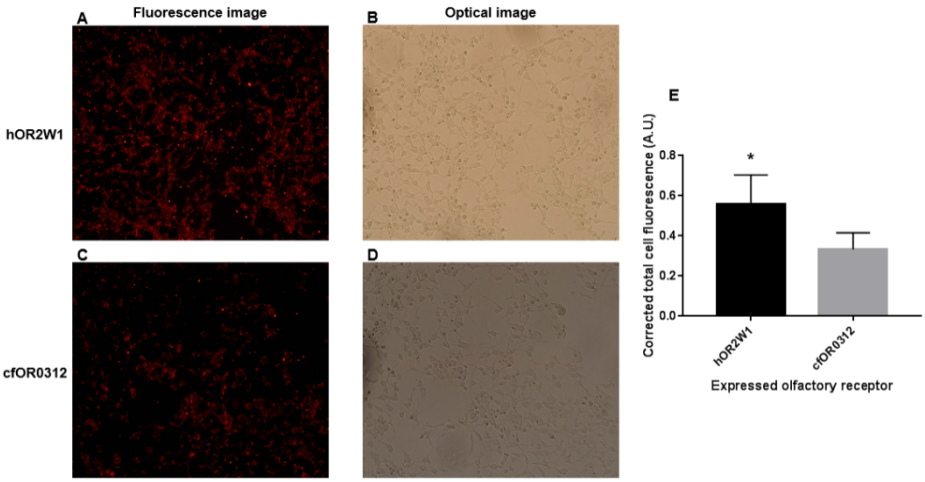

Figure A1.2. Fluorescence images of cells, expressing (A) hOR2W1 proteins and (C) cfOR0312 proteins. Optical images of cells expressing (B) hOR2W1 proteins, and (D) cfOR0312 proteins. Images were taken at 100X amplification. (E) Comparison of OR expression by using the corrected total cell fluorescence (CTCF). Results shown represent the mean ± SD, n = 10 and *p < 0.05.

A1.5 Comparison of human and canine OR sensitivity to hexanal

To investigate sensitivity of human and canine ORs to hexanal, intracellular calcium quantification assay was conducted with Fura-2 AM. Hexanal was serially diluted from 1 M to 10 nM with Ringer's solution, including 10 μM probenecid. To confirm activity of OR-expressing HEK293 cells, 10 μM ATP in Ringer's solution was additionally injected, after injection of hexanal solutions. To avoid leakage of dye during calcium quantification assay, OR-expressing cells were incubated in Ringer's solution including 10 μM probenecid at 37℃ for 1 h after 30 min treatment of 5 μM Fura-2 AM in Ringer's solution. It took 60 s per 1 cycle of assay, and injection timing of hexanal and ATP solution to wells containing OR-expressing cells were 20 s and 45 s. The identical process was repeated in 3 wells, for each concentration of hexanal.

The response of OR was observed through the change in fluorescence intensity. Fura-2 AM is a fluorescent dye which emits fluorescence at different excitation wavelength. When in calcium free condition, Fura-2 AM emits fluorescence at 380 nm excitation wavelength. But when this dye is bound to calcium ions, the fluorescence is generated at 340 nm excitation wavelength. The emission wavelength was 510 nm in both cases. By using the ratio of fluorescence intensities with the excitation at 340 nm (F340) and with the excitation at 380 nm (F380), the response of OR proteins to the agonist can be estimated.

As a result, hOR2W1 and cfOR0312 showed similar levels of response to 1 mM of hexanal (Figure A1.3A and A1.3B). However, in contrast to cfOR0312, hOR2W1 did not show a response, to the concentration lower than 1 μM hexanal (Figure A1.3C and A1.3D). The dose-response curve of human and canine ORs to hexanal was also analyzed (Figure A1.4A and A1.4B). The dose-response curve was calculated with the following equation.

$$\mathrm{Y} = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{(\log EC_{50} - X)}}$$

Y represents the normalized fluorescence intensity, while X means the concentration of hexanal (M) in log scale. Accordingly, it was revealed that the cfOR0312 is more sensitive to hexanal than the hOR2W1 though the expression rate of hOR2W1 was higher than of cfOR0312 on HEK293 cell surface. These findings would support the claim that dogs possess superior olfactory

sensitivity compared to humans. Although we selected the most sensitive human and canine ORs to hexanal as representative ORs, it is not sufficient to generalize olfactory sensitivity of species. To understand factors associated with olfactory sensitivity, behavioral studies as well as more molecular level studies should be conducted.

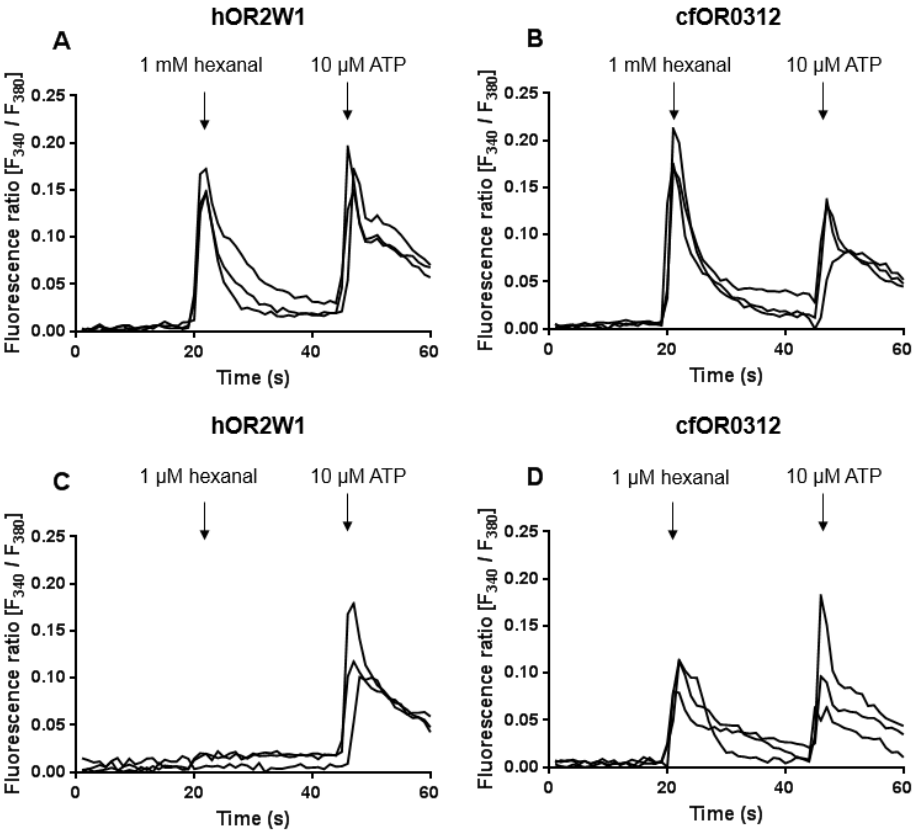

Figure A1.3. Olfactory signals were induced by hexanal and 10 μM ATP solution. Ringer's solution including 1 mM or 1 μM hexanal was added, to HEK293 cells expressing (A, C) hOR2W1 and (B, D) cfOR0312 proteins at 20 s. ATP solution was injected at 45 s in all cases, as a positive control. Fluorescence ratio (F340/F380) was increased by calcium ion influx via olfactory signaling pathways.

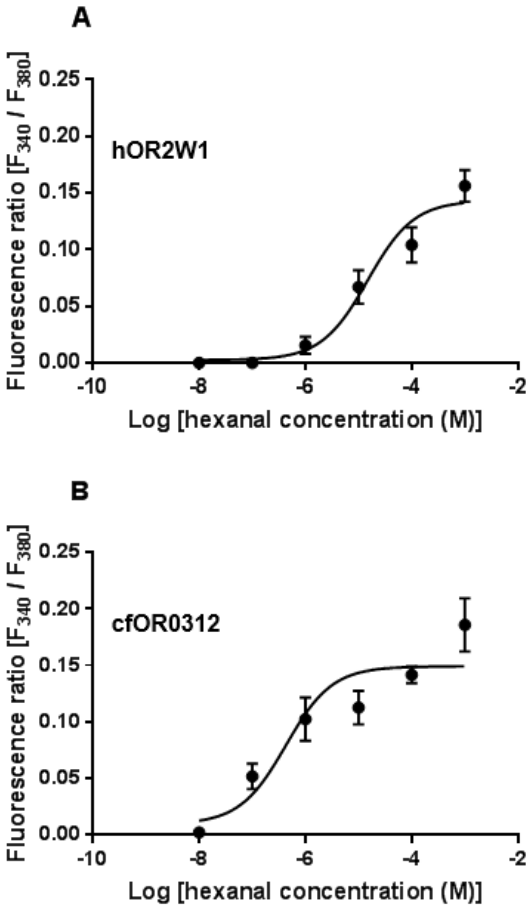

Figure A1.4. Dose-response curve of (A) hOR2W1 and (B) cfOR0312 to hexanal. Each data point indicates the mean, and the error bar indicates standard deviation (n=3-4). The dose-response curve was calculated with the model equation. R square values were 0.94 for (A) and 0.84 for (B).

A1.6 Conclusions

We compared sensitivity of human and canine ORs to hexanal. The hOR2W1 and cfOR0312 genes were amplified, by PCR and inserted to the pcDNA3 vector with Rho-tag. These OR genes were successfully transfected to HEK293 cells, and expression of OR proteins was confirmed by an immunocytochemistry method. The intracellular calcium quantification assay was conducted, to observe the change in fluorescence intensities varying with calcium ion influx induced by olfactory signaling pathways. As a result, it was concluded that cfOR0312 possesses superior sensitivity to hexanal than hOR2W1. The cfOR0312 showed a response to 100 nM hexanal, but hOR2W1 did not show response to the concentration lower than 1 μM hexanal. Results support the hypothesis that the superior olfactory sensitivity of dogs may also depend on higher performance of the olfactory receptor. The comparative evaluation of OR sensitivity at a molecular level would be a major factor in comparing smell sensing ability among species.

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초 록

의료기술과 체계의 발달로 인해 질병으로 인한 조기 사망률은 과거에 비해 크게 줄어들었다. 그러나 암을 비롯한 일부 난치성 질병으로 인한 치사율은 여전히 높은 편이다, 이는 질병이 치명적인 수준까지 발달하기 전에 자각증상을 느끼기 힘들다는 점과 기존의 검진 방법이 특유의 침습적인 방식과 검사 비용 때문에 접근성이 떨어진다는 점에서 비롯된다. 이런 연유로 최신 질병 진단 기술은 접근성의 향상을 추구하는 방향으로 발전하고 있으며, 특히 비 침습적이고 경제적인 방법의 필요성이 대두되고 있다. 대표적인 예시로, 특이적인 휘발성 유기물질을 감지하여 질병을 진단하는 기술은 피나 체액 뿐만 아니라 날숨, 땀, 소변, 침 등을 매개로 와병 여부를 파악할 수 있기에 고통이 수반되지 않는 간단한 진단을 가능케 한다. 특히, 바이오 전자 센서는 카본나노튜브나 그라핀 같은 나노 구조 반도체를 포함한 2 차 변환기에 후각 수용체와 같은 1 차 변환기를 결합하여 우수한 선택도와 민감도를 선보인 바 있다.

본 연구의 목적은 질병 진단용 바이오 전자 센서 제작을 위해 필수적으로 이루어져야 하는 질병 표지물질 선정과, 표지물질 탐지를 위한 후각 수용체 발굴 및 성능 평가이다. 연구 대상으로 선택한 질병은 폐암, 결핵, 그리고 위암이다. 먼저 폐암의 표지물질 발굴과 인간 후각 수용체 탐색이 수행되었다. 폐암 세포주와 정상 폐 세포주를 배양하여 두부공간의 가스 조성을 GC/MS 로 비교하였고, 폐암 세포에서 더 많이 발생하는 휘발성 유기물질 2-에틸헥산올을 특정하였다. 그리고 이 물질을 감지할 수 있는 인간 후각 수용체를 이중 발광 루시퍼레이즈 검정법을 이용하여 탐색하였다. 발굴된 후각 수용체가 폐암 표지물질을 민감하고 선택적으로 감지하는 것을 확인하였으며, 향후 바이오 전자 센서의 1 차 소자로 사용하기 위한 후각 나노베시클 생산 및 성능 평가를 진행하였다.

두 번째 연구에서는 소변에서 발견된 결핵 관련 5 종의 휘발성 유기물질들을 감지할 수 있는 인간 후각 수용체를 탐색하였다. 탐색 과정은 HEK293 세포주에 인간 후각 수용체 유전자와 루시퍼레이즈 리포터 유전자를 형질도입하여 결핵 바이오마커들에 대한 반응성을 확인함으로써 진행되었다. 실험 결과 각각의 결핵 바이오마커에 대한 후각 수용체가 선정되었으며, 그 반응성과 선택도 또한 분석되었다.

세번째로, 위암 환자와 건강한 사람의 날숨 샘플을 다수 채취하여 GC/MS 장비를 이용해 분석하고 비교하였다. 그 결과 위암 환자에게서 상대적으로 많이 발견되는 휘발성 유기물질인 뷰틸산과 프로피온산을 특정하였다. 특히, 날숨 샘플 내에 매우 적은 양이 포함된 표지물질을 빠짐없이 분석하기 위해 휘발성 유기물질 채취 및 농축 수단으로 고체 미세추출 (SPME) 섬유를 활용하였다. 선정한 휘발성 유기물질의 표지물질로서의 신뢰도를 제고하기 위해, 전체 자료의 통계 분석 과정을 통해 날숨 내의 표지물질 포함량을 기준으로 환자 여부를 구분짓는 진단 모델을 구축하고 그 민감도와 선택도를 산출하였다. 추가적으로, 향후 진행할 날숨을 대상으로 한 위암 진단용 바이오 전자 센서 제작을 위해, 뷰틸산과 프로피온산을 감지한다고 알려진 인간 후각 수용체 2 종의 반응성과 선택도를 분석하였다.

질병 진단기술 개발은 인류의 보편적 복지와 평균수명 연장을 위하여 필연적으로 이루어져야 하는 과정이다. 질병 특이적 휘발성 유기물질을 대상으로 삼는 진단 방식은 차세대 진단기술로써 학계에서 주목받고 있으며, 세계 각지에서 활발하게 연구되고 있다. 본 논문에서는 몇 가지 질병 특이적 휘발성 유기물질이 신규 발굴되었으며, 또한 기존에 알려진 질병 표지물질을 감지하는 능력을 가진 후각 수용체를 탐색하고 그 기능성을 확인하였다. 상술한 연구 성과들이 민감하고 선택적인 질병 진단용 생체 소자 개발에 유용하게 활용되길 기대한다.

주요어: 휘발성 유기물질, 표지물질, GC/MS, SPME, 후각 수용체, 바이오 전자 센서

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