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Discovery and Validation of Salivary Extracellular RNA Biomarkers for Noninvasive Detection of Gastric Cancer

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Abstract

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BACKGROUND: Biomarkers are needed for noninvasive early detection of gastric cancer (GC). We investigated salivary extracellular RNA (exRNA) biomarkers as potential clinical evaluation tools for GC.

METHODS: Unstimulated whole saliva samples were prospectively collected from 294 individuals (163 GC and 131 non-GC patients) who underwent endoscopic evaluation at the Samsung Medical Center in Korea. Salivary transcriptomes of 63 GC and 31 non-GC patients were profiled, and mRNA biomarker candidates were verified with reverse transcription quantitative real-time PCR (RT-qPCR). In parallel, microRNA (miRNA) biomarkers were profiled and verified with saliva samples from 10 GC and 10 non-GC patients. Candidate biomarkers were validated with RT-qPCR in an independent cohort of 100/100 saliva samples from GC and non-GC patients. Validated individual markers were configured into a best performance panel.

RESULTS: We identified 30 mRNA and 15 miRNA candidates whose expression pattern associated with the presence of GC. Among them, 12 mRNA and 6 miRNA candidates were verified with the discovery cohort by RT-qPCR and further validated with the independent cohort (n = 200). The configured biomarker panel consisted of 3 mRNAs (*SPINK7*, *PPL*, and *SEMA4B*) and 2 miRNAs (*MIR140-5p* and *MIR301a*), which were all significantly down-regulated in the GC group, and yielded an area under the ROC curve (AUC) of 0.81 (95% CI, 0.72–0.89). When combined with demographic factors, the AUC of the biomarker panel reached 0.87 (95% CI, 0.80–0.93).

CONCLUSIONS: We have discovered and validated a panel of salivary exRNA biomarkers with credible clinical performance for the detection of GC. Our study demonstrates the potential utility of salivary exRNA biomarkers in screening and risk assessment for GC.

Gastric cancer (GC)[8] is the fourth most common cancer diagnosed globally and the third leading cause of cancer-related deaths (1). It is the leading cancer type diagnosed in East Asian countries (2). In Korea, genetics (3), diets containing salted and preserved foods (4), smoking (5), and a high prevalence of *Helicobacter pylori* infections (6) play a role in a large percentage of the population with GC. In Korea, most early-stage GCs are identified in asymptomatic individuals (74.2%–78.1%) compared with symptomatic individuals (25.9%–35.7%) (7). Once the disease progresses and results in serious symptoms and complications, the prognosis is poor and the survival rate decreases from approximately 65% (when found at an early stage) to <20% (8). In 1999, owing to the high prevalence of GC, the National Cancer Screening Program in Korea implemented an ongoing early detection program that recommends everyone over the age of 40 years undergo an upper endoscopy every other year (9). However, an upper endoscopy for GC detection is costly, time-consuming, and invasive. Since the screening program began, <30% of the targeted population participated (10). Thus, there is a need for predictive biomarkers that can be used as a credible screening tool for early detection of GC. These biomarkers are highly desirable to improve the outcome of the disease and reduce unnecessary endoscopies.

[8]Nonstandard abbreviations: GC, gastric cancer; ROC, receiver operating characteristic; AUC, area under the ROC curve; miRNA, microRNA; RT-qPCR, reverse transcription quantitative real-time PCR; exRNA, extracellular RNA; PRoBE, prospective-specimen collection and retrospective blinded evaluation.

Several studies have looked for potential biomarkers in serum as a noninvasive method for screening GC. The serum pepsinogens I and II at low concentrations and their ratio have been considered indicators for preneoplastic gastric lesions, but results have varied depending on the location of cancer and the cutoff values used in different studies (11). The most frequently used gastric tumor markers, such as carcinoembryonic antigen, CA19-9, CA-50, and CA72-4, have reported area under the ROC curve (AUC) values of 0.54 to 0.73; thus, they are not sensitive or specific enough to screen for GC patients (12, 13). In addition to proteins, various types of RNA are emerging biomarkers for GC. A few studies have performed systematic microRNA (miRNA) profiling of blood samples from patients with GC. The expression patterns of *MIR221*[9], *MIR744*, and *MIR376c* in serum showed value as biomarkers to distinguish GC patients from healthy individuals (14). Plasma miRNA biomarkers for GC were found by reverse transcription quantitative real-time PCR (RT-PCR), and the AUCs were 0.65 to 0.75 for *MIR185, MIR20a*, *MIR210, MIR25*, and *MIR92b* (15). In another study, *MIR181a-1* and *KAT2B* mRNA were identified as a combined predictor with AUC >0.95 (16). These studies suggested RNAs as potential biomarkers in the diagnosis and prognosis of GC; however, no study with definitive validation of these biomarkers in a large enough cohort has been conducted. Thus, the value of these biomarkers needs to be further confirmed in human GC patients.

Salivary extracellular RNA (exRNA) biomarkers including miRNAs have been developed for detecting various local and systemic diseases such as oral cancer (17, 18), Sjögren syndrome (19), pancreatic cancer (20), breast cancer (21), and lung cancer (22). In this study, we developed salivary exRNA biomarkers for GC detection in a Korean high-risk population based on prospective specimen collection (before the tumor diagnosis) and retrospective blinded evaluation (PRoBE) guidelines (23).

Materials and Methods

SAMPLE COLLECTION AND STUDY DESIGN

This study was performed at the University of California, Los Angeles (US) and Samsung Medical Center (South Korea) with approval from the institutional review boards from both institutions (UCLA IRB 06–07-018–11, SMC IRB 2008–01-028 –016). The study design followed the principles of the PRoBE design (23). All study participants were recruited from the Samsung Medical Center, and 294 saliva samples (163 GC and 131 non-GC) were prospectively collected before endoscopic examination. The detailed patient enrollment and cell-free saliva collection procedure can be found in the Methods section of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue10. We have performed sample randomization based on the demographic information of all patients. The age, ethnicity, and alcohol consumption of healthy

[9]Human Genes: *MIR221*, microRNA 221; *MIR744*, microRNA 744; *MIR376c*, microRNA 376c; *MIR185*, microRNA 185; *MIR20a*, microRNA 20a; *MIR210*, microRNA 210; *MIR25*, microRNA 25; *MIR92b*, microRNA 92b; *MIR181a-1*, microRNA 181a-1; *KAT2B*, lysine acetyltransferase 2B; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, actin beta; *S100A10*, S100 calcium binding protein A10; *ANXA1*, annexin A1; *CSTB*, cystatin B; *KRT6A*, keratin 6A; *ERO1A*, endoplasmic reticulum oxidoreductase 1 alpha; *PPL*, periplakin; *SPINK7*, serine peptidase inhibitor, Kazal type 7; *RANBP9*, RAN binding protein 9; *KRT4*, keratin 4; *CD24*, CD24 molecule; *SEMA4B*, semaphorin 4B; *EIF3G*, eukaryotic translation initiation factor 3 subunit G; *MIR140-5p*, microRNA 140-5p; *MIR374a*, microRNA 374a; *MIR454*, microRNA 454; *MIR15b*, microRNA 15b; *MIR28-5p*, microRNA 28-5p; *MIR301a*, microRNA 301a; *ECRG2*, esophagus cancer-related gene 2.

individuals used in this study were balanced in the patient group, as shown in Table 1. Two demographic factors, smoking and the presence of *H. pylori*, could not be balanced between the groups, as these are known risk factors for GC (5, 24).

The biomarker development study consisted of 2 parts: discovery and validation. The first part was the discovery and verification phase of biomarkers using 2 different platforms: transcriptomic and miRNA (Fig. 1). The salivary transcriptomes of 63 GC samples and 31 non-GC controls (Table 1) were profiled using Affymetrix HG U133 + 2.0 microarrays. The identified exRNA candidates were verified by RT-qPCR using all 94 of the original samples. In the discovery phase for the miRNA biomarkers, 10 early-stage GC samples and 10 non-GC controls were selected (Table 1). The salivary miRNAs of these samples (n = 20) were profiled using the TaqMan miRNA array (Applied Biosystems). The miRNA candidates were verified using TaqMan miRNA assay (Thermo Scientific). The second part of the study was to validate these verified exRNA biomarker candidates with exRNA samples extracted from an independent cohort of 100 GC and 100 non-GC saliva samples (Table 2). The cohort was not balanced for demographics on sex and smoking history but more accurately reflected the diagnostic setting where our proposed final model could be implemented. Table 1 in the online Data Supplement shows the histological classification of GC individuals in the validation cohort.

SALIVARY TRANSCRIPTOMIC PROFILING AND DATA ANALYSIS

Total RNA was isolated from 300 μL of saliva supernatant using the miRNeasy micro kit (QIAGEN). The method to avoid RNase contamination can be found in the Methods section of the online Data Supplement. The extracted RNA was treated with DNase I (Ambion) to remove contaminating DNA. The quality of salivary mRNA was evaluated by detecting expression levels of a saliva internal reference gene (*GAPDH*) using RT-qPCR (25). Isolated salivary mRNA (approximately 10 ng) was amplified using the RiboAmp RNA Amplification kit (Molecular Devices) and biotin-labeled using GeneChip Expression 3′-Amplification Reagents for in vitro transcription labeling (Affymetrix). Biotin-labeled complementary RNA (approximately 20 μg) was subsequently fragmented and sent to the University of California, Los Angeles microarray core facility for chip hybridization and scanning. The Affymetrix Human Genome U133 Plus 2.0 Array, which represents >47 000 transcripts and variants, was used for the salivary transcriptomic profiling. The microarray data have been uploaded to the GEO database (access no. GSE64951) based on the Minimum Information About a Microarray Experiment guidelines (26)

mRNA BIOMARKER VERIFICATION USING RT-qPCR

The selected candidate mRNA biomarkers (12) generated by microarray profiling were verified by nested RT-qPCR (RT-PCR followed by a separate SYBR green qPCR) on the same set of samples used for the microarray analysis (n = 94). The qPCR primers were designed using Primer3 software and synthesized by Sigma-Genosys after performing a Primer-BLAST search (27). The primer sequences were designed to avoid any known single-nucleotide polymorphism region in the target gene. All the amplicons were intron spanning. The RT-qPCR assay followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiment guidelines and was performed in duplicate with

each biomarker candidate (28). The detailed protocol can be found in the Methods section of the online Data Supplement. The specificity of the PCR product for each gene was confirmed with melting curve analysis and 3% agarose gel analysis. We calculated ΔCq by subtracting the Cq value of the housekeeping gene (*GAPDH/ACTB*) from the raw Cq value of each biomarker candidate. The gene accession numbers and primer sequences used for transcriptomic biomarker verification and validation are shown in Table 2 of the online Data Supplement.

SALIVARY miRNA PROFILING

Total RNA was extracted from 300 μL of saliva supernatant using the mirVana PARIS extraction kit (Ambion). On-column DNase treatment (Qiagen) was used to remove contaminating DNA during RNA extraction. Total RNA (3 ng) was converted to complementary DNA using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems). Two different sets of stem-loop RT primers (human pool A and human pool B) were used (Megaplex RT primers, Applied Biosystems). After reverse transcription, the RT product was preamplified using TaqMan PreAmp Master Mix (Applied Biosystems) and Megaplex PreAmp primers (Applied Biosystems). The preamp product was not diluted before miRNA quantification. The TaqMan Human miRNA array set version 3.0 (Applied Biosystems) and TaqMan Universal PCR Master Mix, no AmpErase uracil *N*-glycosylase were used for miRNA quantification. All reactions were performed on a 7900HT Fast Real-Time PCR System containing a special cardholder (Applied Biosystems). Data were analyzed using RQ Manager software version 1.2 and DataAssist software version 3.0 (Applied Biosystems). Similarly, the ΔCq value was computed using RNA polymerase III transcribed U6 small nuclear RNA as the reference gene.

VERIFICATION AND VALIDATION OF SALIVARY miRNA BIOMARKERS

The biomarker candidates generated by the TaqMan miRNA array profiling were verified by TaqMan miRNA assays (Applied Biosystems) on the same set of samples used for the miRNA array analysis (n = 20). TaqMan miRNA assays containing specific miRNA genes were ordered from Applied Biosystems. The protocol was like that recommended by the manufacturer for creating custom RT and preamplification pools using TaqMan miRNA assays. There was no dilution of sample before the real-time PCR reaction. The qPCR reactions for each candidate miRNA were performed in duplicate on a Roche LightCycler 480 II (Roche). The average threshold cycle (Cq) was examined, and U6 small nuclear RNA was used as the reference gene for normalizing the data. The TaqMan miRNA assay was also used to assay miRNAs in the validation cohort.

STATISTICAL ANALYSIS

Initial analyses summarized demographic characteristics within each cohort. Next, χ^2 tests and *t*-tests were used to compare demographic characteristics between cancer and noncancer participants within cohorts.

Microarray analysis: The CEL files from all data sets were imported into the statistical software R 3.0.2 (29) using Bioconductor 2.2 (30). The data preprocessing was performed using the Probe Logarithmic Intensity Error Estimation expression measures after

background correction and quantile normalization for each microarray expression data set. Probe set-level quantile normalization was performed across all samples. Finally, for every probe set, the Wilcoxon rank-sum test was used to compare gene expression between GC patients and non-GC controls.

The candidate salivary mRNA and miRNA biomarkers met the following criteria: (*a*) *P* value from Wilcoxon test <0.05 and (*b*) fold change >1.2. The top 30 ranking mRNA and 15 ranking miRNAs with the smallest *P* values were selected for verification. In the RT-qPCR verification step, ΔCq values for mRNA and miRNA were compared between groups using the Wilcoxon rank-sum test. Twelve mRNA and 6 miRNA markers with $P < 0.05$ were included in the panel for evaluation in the validation step.

VALIDATION AND MODEL BUILDING

For each of the 12 mRNA and 6 miRNA candidates chosen from the discovery set, the Wilcoxon rank-sum test was used to compare markers between groups in the validation set of patients (100 GC vs 100 non-GC individuals). First, the Wilcoxon rank-sum test was used to compare ΔCq transformed values for each marker between the GC and non-GC groups. Next, we created a multiple logistic model to identify the best combination of markers that could discriminate GC from non-GC samples.

We used the LASSO variable selection technique/estimation to construct the logistic regression model (31). The tuning parameter (λ) was chosen via 10-fold cross-validation with the GLMnet package in R. The diagnostic ability of the model was assessed using the AUC computed based on the predicted probabilities from the model. Statistical analyses were carried out using R 3.0.2 and SPSS V22 (IBM Corp). Values (in tables) are reported as mean (SD), and *P* values <0.05 were considered statistically significant.

Results

DISCOVERY OF SALIVARY TRANSCRIPTOMIC CANDIDATE BIOMARKERS

Gene expression profiles of saliva samples from GC patients (n = 63) and non-GC controls (n = 31) were examined in the discovery phase using Affymetrix Human Genome U133 Plus 2.0 Array (Fig. 1). To ensure accuracy of the microarray profiling, the quantity and quality of RNA in each saliva sample were assessed. On average, 117.51 ± 70.67 ng (n = 94) of total RNA was obtained from 300 μL of saliva supernatant. There was no significant difference in the total RNA isolated between the GC patients and non-GC controls ($P = 0.39$; n = 94). The RT-qPCR results of a saliva internal reference gene *GAPDH* in all saliva samples showed no significant difference in expression levels between GC patients and non-GC controls ($P = 0.71$; n = 94). A consistent amplification magnitude was obtained after 2 rounds of amplification, yielding an average of 58.58 ± 14.76 μg of biotinylated complementary RNA. There was no significant difference in the yield of complementary RNA between GC patients and non-GC controls ($P = 0.23$; n = 94).

Expression microarray results revealed 38 extracellular mRNAs significantly up-regulated and 2601 extracellular mRNAs significantly down-regulated in the saliva of GC patients when compared with the saliva of non-GC controls (n = 94; $P < 0.05$; fold change > 1.2). A

heat map built from the microarray analysis of the top 150 genes resulted in an unadjusted *P* value cutoff of 0.002, revealing a potentially different saliva profile between the 2 groups (see Fig. 1 in the online Data Supplement).

VERIFICATION OF mRNA CANDIDATE BIOMARKERS FOR GC DETECTION

The candidate mRNA markers from microarray profiling were verified before validation. The top 30 ranking mRNA candidates (25 down-regulated and 5 up-regulated) with the smallest *P* values were selected for verification. Then, RT-qPCR was performed to verify the results on the discovery sample set (n = 94) and confirmed the differential RNA expression level of 12 of the 30 exRNAs, which were consistent with the microarray data and showed significant differences ($P < 0.05$) between GC patients and non-GC controls. Using *GAPDH* gene, the Cq from the GC patients was 24.85 ± 1.53, whereas the Cq from the non-GC controls was 25.03 ± 2.32, showing no significant difference ($P = 0.65$). As shown in Table 3 of the online Data Supplement, 11 down-regulated exRNAs (*S100A10, ANXA1, CSTB, KRT6A, ERO1A, PPL, SPINK7, RANBP9, KRT4, CD24*, and *SEMA4B*) and 1 up-regulated exRNA (*EIF3G*) were verified ($P < 0.05$; n = 94). The expression patterns of the verified biomarkers were consistent with the microarray profiling and exhibited AUC values of 0.63 to 0.74.

SALIVARY miRNA EXPRESSION PROFILES, CANDIDATE DISCOVERY, AND VERIFICATION

The miRNA expression profiles of 10 early-stage GC patients (stage 1a or 1b) and 10 non-GC controls were used for miRNA discovery. Both TaqMan Human miRNA A and B array version 3.0 cards were used to profile each sample (40 cards total). Before data normalization, only miRNAs with Cq values <35 in at least 80% of samples (16 of 20 patients) were included to ensure accuracy of miRNA verification by RT-qPCR. The numbers of detectable miRNAs between the saliva of GC patients and non-GC controls were similar (218 ± 3). Using the aforementioned criteria, 15 miRNA candidates (12 down-regulated and 3 up-regulated) were selected for verification by RT-qPCR using the discovery phase sample set. For the U6 small nuclear RNA reference gene, the Cq values acquired were 18.79 ± 1.37 from the GC patients and 18.67 ± 1.90 ($P = 0.87$) from the non-GC controls. RT-qPCR confirmed that the relative expression levels of 6 miRNAs were consistent with the TaqMan miRNA array data and showed significant differences ($P < 0.05$) between GC patients and non-GC controls. As shown in Table 4 of the online Data Supplement, 6 down-regulated miRNAs (*MIR140-5p, MIR374a, MIR454, MIR15b, MIR28-5p*, and *MIR301a*) were verified. These miRNAs exhibited AUC values of 0.79 to 0.88.

VALIDATION OF mRNA AND miRNA CANDIDATE BIOMARKERS

From the top 30 ranking mRNA and 15 ranking miRNAs candidates, the 12 verified mRNA candidates and 6 verified miRNA candidates were chosen for further validation using an independent cohort (100 GC patients and 100 matched non-GC controls). As shown in Table 3, 9 of the 12 mRNAs were validated, including *ANXA1, CD24, CSTB, ERO1A, KRT4, KRT6A, PPL, S100A10*, and *SPINK7* ($P < 0.05$), yielding AUC values of 0.59 to 0.64 (Table 3). Four of 6 miRNA candidates were validated, including *MIR140-5p, MIR374a, MIR454*, and *MIR15b*. All 4 miRNA biomarkers showed significant differences between

GC patients and non-GC controls ($P < 0.05$; n = 200), yielding AUC values of 0.63 to 0.70 (Table 3).

PREDICTION MODEL CONSTRUCTION USING VALIDATED SALIVARY exRNA BIOMARKERS

From the 12 mRNA and 6 miRNA candidates, 3 mRNA biomarkers (*SPINK7, PPL*, and *SEMA4B*) and 2 miRNA markers (*MIR140-5p* and *MIR301a*) selected by the LASSO procedure yielded an AUC value of 0.81 (95% CI, 0.72–0.89) (Fig. 2, black dashed ROC curve). The point on the ROC curve that maximizes sensitivity and specificity results in a test being 75% sensitive and 83% specific. Setting the sensitivity at 80% or 90% yields specificity estimates of 54% and 40%, respectively. To assess the prognostic ability of our markers, we constructed a demographic characteristic-only model from our GC database repository. The model applied to our validation data set resulted in an AUC of 0.69 (95% CI, 0.59–0.79) (Fig. 2, dark gray solid ROC curve) with coefficients summarized in Table 5 of the online Data Supplement (smoking, sex, age). The combination of assessments of the exRNA panel and the demographic variables (smoking, sex, and age) provided an AUC of 0.87 (95% CI, 0.80–0.93) (Fig. 2, gray long dashed ROC curve; see also Table 6 in the online Data Supplement). The calibration tests of 3 presented models using the Hosmer–Lemeshow test is shown in Table 7 of the online Data Supplement. All 3 models have $P > 0.05$, indicating no significant lack of calibration. The comparison of the AUCs of these 3 models was performed with DeLong's test (see Table 8 of the online Data Supplement). Only the markers plus demographics model (AUC = 0.87) vs demographics-only model (AUC = 0.69) showed a significant difference in the Δ AUC. The point on the ROC curve of this model with maximum sensitivity and specificity is sensitivity = 82% and specificity = 77%. The positive predictive value was 82% and the negative predictive value was 77%. If we set a threshold with high sensitivity (90%), the respective specificity, positive predictive value, and negative predictive value are 65%, 76%, and 84%, respectively.

Discussion

This biomarker development study identified salivary biomarkers that can be definitively validated for GC detection. Nine of the 30 (30%) top-ranking salivary mRNA candidates and 4 of the 15 (27%) top-ranking miRNA salivary biomarkers were discovered based on a prospective clinical design, compliant with the PRoBE guidelines for biomarker development, and were validated. The discovered salivary mRNA and miRNA biomarkers were first individually validated in a cohort of 100 GC participants and 100 non-GC controls to yield the best performance panel of 3 mRNAs (*SPINK7, PPL*, and *SEMA4B*) and 2 miRNAs (*MIR140-5p* and *MIR3014*) with an AUC value of 0.81. Combined with demographic variables, the performance of the panel reached an AUC of 0.87 (95% CI, 0.80–0.93). We also analyzed whether validated biomarker candidates could distinguish different stages of GC. As shown in Table 9 of the online Data Supplement, only expression of *PPL* showed significant differences between early and late stages of GC.

One of the limitations of this study is that all participants included in this study were from a Korean cohort; the performance of this panel in other populations, especially in Western

countries, needs to be further determined. Another limitation is that our model coefficients were constructed and validated on the same cohort. Thus, a further validation with an independent cohort from a multisite study would be required before clinical usage. *H. pylori* is known as 1 of the most potent risk factors for GC, although we could not include it in our final model because the screening population data were not available for much of the validation cohort. We do not think the absence of *H. pylori* index would be a confounding factor for the performance of our markers, but it may be something to further explore in a follow-up study.

Intriguingly, the most significant discriminative marker *SPINK7*, which is also named *ECRG2* (esophagus cancer-related gene 2), was found dramatically down-regulated in primary esophageal squamous carcinoma (32). It is a tumor-suppressor gene that inhibits invasion of cancer cells through the urokinase-type plasmin activator receptor/β1 integrin pathway (33). Periplakin (*PPL*) has been reported to be down-regulated in esophageal squamous carcinoma and urothelial carcinoma (34). It also can act as a tumor suppressor in colon cancer progression (35). *SEMA4B* can work as a tumor suppressor to inhibit the invasion of non–small cell lung cancer through the PI3K/AKT pathway (36). Recently, *MIR140* was found significantly decreased in breast cancer and non–small cell lung cancer tissues and cell lines. It also functions as a tumor suppressor in these cancers (37). The discovery of these down-regulated tumor-suppressor genes in saliva from GC patients may reflect their tumor-suppressor functions in GC tissues or just the indirect reactions of the human body to GC. This also needs to be determined in future study.

It is notable that the AUCs of carcinoembryonic antigen and CA19-9, which are currently regarded as the most valuable serum protein markers for the diagnosis of early-stage GC, were 0.73 and 0.68, respectively (12, 13). The performance of these validated salivary exRNA biomarkers is better than that of many existing and clinically used biomarkers. Thus, the merit of this study is not necessarily the development of validated biomarkers with outstanding performance; rather, it is the development of salivary biomarkers that are validated with discriminatory performance. This is of value to the emerging field of salivary diagnostics, as all efforts hinge on the biomarkers achieving regulatory approval by surviving definitive clinical validation trials. This study assures that salivary biomarkers, when properly developed, can be definitively validated for translational and clinical utilities.

An important rationale for this study was to determine the translational validity of salivary biomarkers for systemic disease detection. To date, no salivary biomarkers have been developed de novo and then definitively validated in a specific clinical context *(23*). Our study is the first to profile exRNA in saliva samples from individuals with a systemic disease and advance toward definitive validation. The validated salivary biomarkers, which are discriminatory for GC, can be used for screening GC patients and reduce unnecessary endoscopies. Our findings enhance the prospect for salivary diagnostics in the detection of systemic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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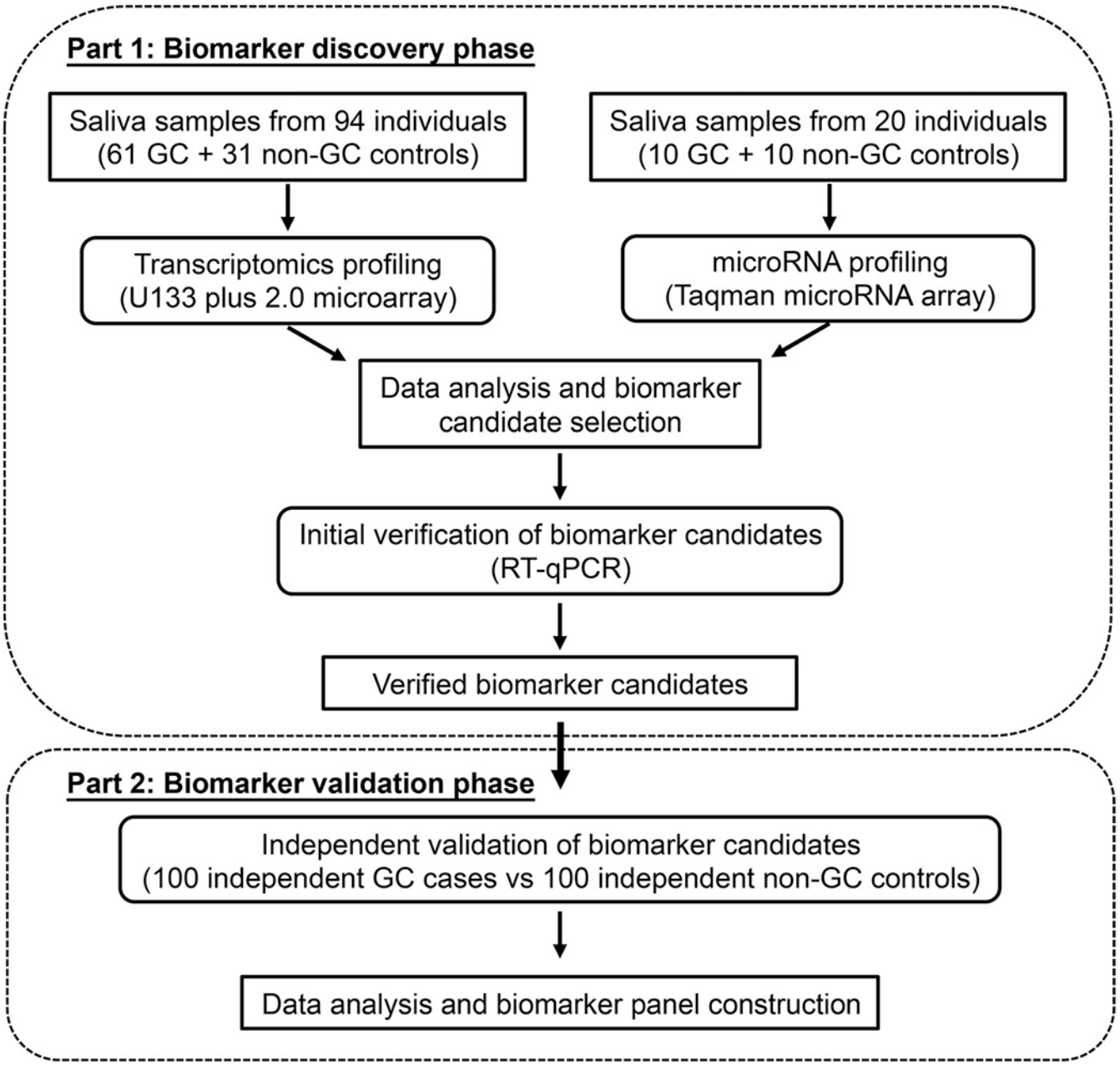

Fig. 1.
Schematic diagram of the study design for the 2 phases of salivary exRNA biomarker development for GC.

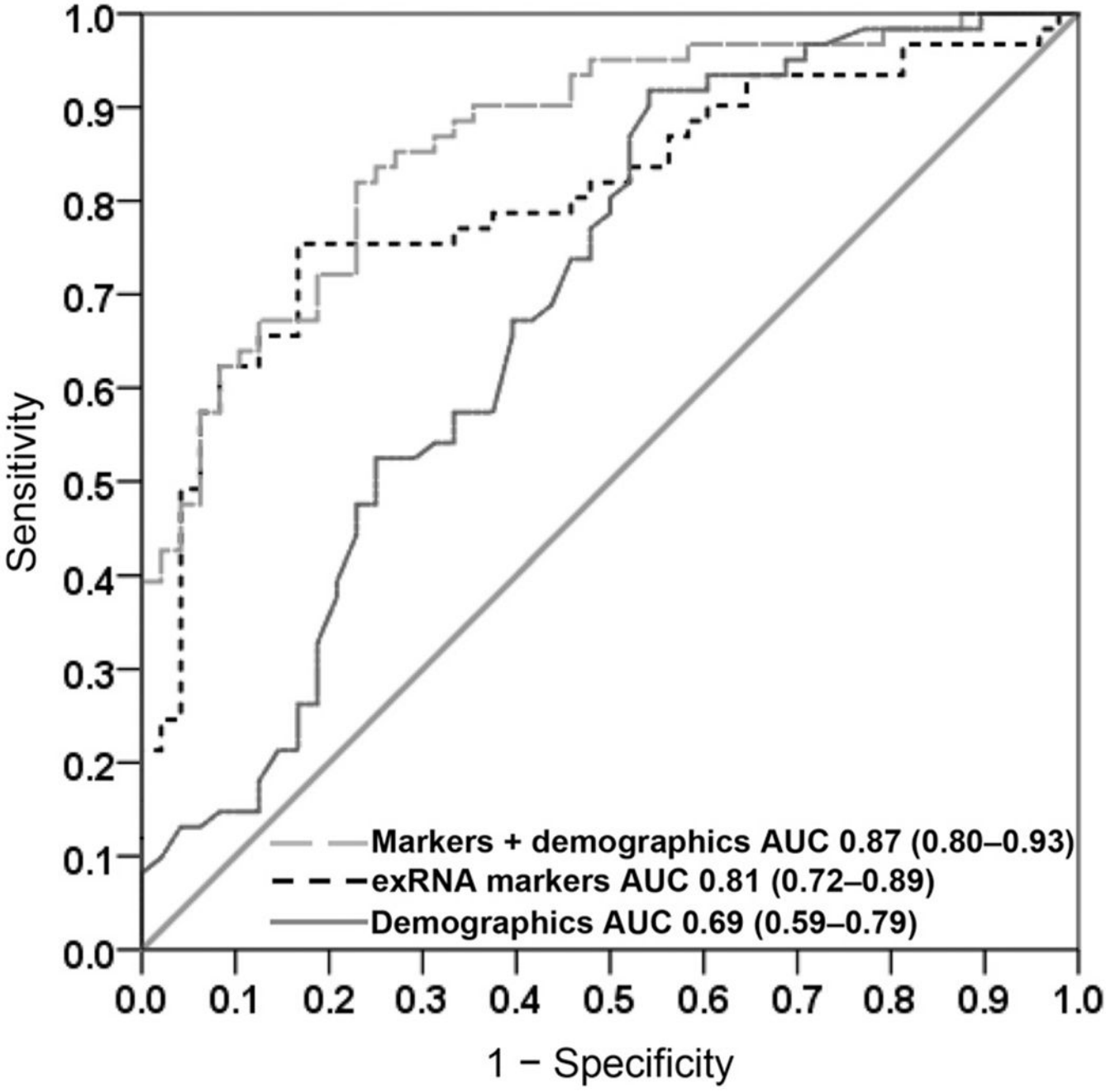

Fig. 2. Clinical utility of 3 salivary mRNA biomarkers (*SPINK7*, *PPL*, and *SEMA4B*) and 2 miRNA biomarkers (*MIR140-5p* and *MIR301a*) combinations.

AUC values of ROC curves computed with 5 biomarkers (black dashed curve), demographic characteristics (dark gray solid curve), and combination of biomarkers plus demographic information (gray long dash curve) are 0.81, 0.69, and 0.87, respectively.

Table 1.

Demographic information on subjects in the discovery phase.

		Transcriptomic biomarker discovery phase[a]			miRNA biomarker discovery phase[b]		
Demographic variable	**Characteristic**	**GC (n = 63)**	**Non-GC (n = 31)**	***P* value**	**GC (n = 10)**	**Non-GC (n = 10)**	***P* value**
Age, years	Mean ± SD	56.2 ± 11.1	54.8 ± 10.4	0.56	58.4 ± 7.6	52.4 ± 7.9	0.10
Gender, n (%)	Male	43 (68.3)	13 (41.9)	0.02	6 (60.0)	4 (40.0)	0.66
	Female	20 (31.7)	18 (59.1)		4 (40.0)	6 (60.0)	
Ethnicity, n (%)	Asian	63 (100)	31 (100)		10 (100)	10 (100)	
Smoking, n (%)		27 (42.8)	5 (16.1)	0.01	3 (30.0)	0 (0.0)	0.21
Drinking, n (%)		28 (44.4)	9 (29.0)	0.15	4 (40.0)	5 (50.0)	1.00
H. pylori, n (%)		37 (58.7)	8 (25.8)	0.003	5 (50.0)	4 (40.0)	1.00

[a] Transcriptomic profiling (n = 94).

[b] miRNA profiling (n = 20).

Table 2.

Demographic information on subjects in the validation phase (n = 200).

		Validation phase		
Demographic variable	**Characteristic**	**GC (n = 100)**	**Non-GC (n = 100)**	***P* value**
Age, years	Mean ± SD	51.9 ± 9.2	55.7 ± 8.1	0.003
Gender, n (%)	Male	79 (79.0)	47 (47.0)	<0.001
	Female	21 (21.0)	53 (53.0)	
Ethnicity, n (%)	Asian	100 (100)	100 (100)	
Smoking, n (%)		66 (44.0)	33 (33.0)	<0.001
Drinking, n (%)		55 (55.0)	NA	
H. pylori, n (%)		NA[a]	NA	

[a]NA, not available.

Table 3.

Quantitative RT-qPCR results and ROC-plot AUC values of the 12 mRNA and 6 miRNA biomarker candidates in saliva.

	GC (n = 100) vs non-GC control (n = 100) ΔCq			
Gene	**GC, mean (SD)**	**Non-GC, mean (SD)**	**P value[a]**	**AUC (95% CI)**
ANXA1	−2.58 (2.07)	−3.36 (1.63)	0.008	0.61 (0.53, 0.69)
CD24	1.20 (1.90)	0.32 (1.66)	0.001	0.63 (0.56, 0.71)
CSTB	−2.83 (2.15)	−3.74 (1.79)	0.004	0.62 (0.54, 0.70)
EIF3G	6.98 (3.08)	7.08 (3.21)	0.945	0.50 (0.42, 0.58)
ERO1A	4.53 (2.07)	3.70 (1.96)	0.002	0.63 (0.55, 0.71)
KRT4	−2.28 (2.35)	−3.02 (2.00)	0.035	0.59 (0.51, 0.67)
KRT6A	−0.34 (2.34)	−1.21 (2.15)	0.001	0.63 (0.56, 0.71)
PPL	1.08 (2.23)	0.34 (2.20)	0.007	0.61 (0.53, 0.69)
RANBP9	4.26 (3.11)	3.56 (2.77)	0.157	0.56 (0.48, 0.64)
S100A10	2.21 (2.02)	1.55 (2.04)	0.006	0.61 (0.54, 0.69)
SEMA4B	11.47 (3.98)	10.57 (4.14)	0.149	0.56 (0.48, 0.64)
SPINK7	2.37 (2.72)	1.18 (1.98)	0.001	0.64 (0.56, 0.72)
MIR140-5p	1.54 (3.68)	−1.08 (3.27)	<0.001	0.70 (0.63, 0.78)
MIR374a	6.95 (5.69)	4.26 (4.59)	<0.001	0.65 (0.57, 0.73)
MIR454	4.61 (3.40)	3.14 (3.40)	0.003	0.63 (0.55, 0.70)
MIR15b	2.92 (3.52)	1.00 (3.42)	<0.001	0.65 (0.57, 0.72)
MIR28-5p	5.15 (4.17)	3.59 (3.94)	0.024	0.59 (0.51, 0.67)
MIR301a	8.46 (4.17)	6.95 (3.82)	0.01	0.61 (0.53, 0.69)

[a]All 15 biomarker candidates with $P < 0.05$ have q values (FDR-adjusted P values) of also <0.05.