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Research article

Development of prediction models for the sensitivity of oral squamous cell carcinomas to preoperative S-1 administration

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ABSTRACT

S-1 is an anticancer agent that is comprised of tegafur, gimeracil, and oteracil potassium, and is widely used in various carcinomas including oral squamous cell carcinoma (OSCC). Although an established prediction tool is not available, we aimed to develop prediction models for the sensitivity of primary OSCC cases to the preoperative administration of S-1.

We performed DNA microarray analysis of 95 cases with OSCC. Using global gene expression data and the clinical data, we developed two different prediction models, namely, model 1 that comprised the complete response (CR) + the partial response (PR) versus stable disease (SD) + progressive disease (PD), and model 2 that comprised responders versus non-responders. Twelve and 18 genes were designated as feature genes (FGs) in models 1 and 2, respectively, and, of these, six genes were common to both models. The sensitivity was 96.3%, the specificity was 91.2%, and the accuracy was 92.6% for model 1, and the sensitivity was 95.6%, the specificity was 85.2%, and the accuracy was 92.6% for model 2. These models were validated using receiver operating characteristic analysis, and the areas under the curves were 0.967 and 0.949 in models 1 and 2, respectively. The data led to the development of models that can reliably predict the sensitivity of patients with OSCC to the preoperative administration of S-1. The mechanism that regulates S-1 sensitivity remains unclear; however, the prediction models developed provide hope that further functional investigations into the FGs will lead to a greater understanding of drug resistance.

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1. Introduction

Indications for cancer chemotherapy are difficult to assess, because the mechanisms underlying resistance to anticancer agents are not fully understood. If less effective anticancer agents are chosen, patients do not benefit sufficiently from treatment and they just suffer from severe adverse events, leading to poor outcomes. Thus, a reliable chemosensitivity prediction tool that can indicate whether or not an anticancer agent will be effective is of crucial importance. Following recent innovations in genetic analysis, some clinical prediction tools that use gene expression profiles have been developed [1, 2]. For example, using array-based comparative genomic hybridization data, Korkola et al. developed prediction models for survival in patients with non-seminomatous germ cell tumors who had been treated with cisplatin-based chemotherapy, and they showed that 64–79.6% of patients were correctly classified [3]. Tsunashima et al. developed a multigene expression-based prediction model for a pathological response to neoadjuvant chemotherapy in breast cancer cases [4].

S-1 is an anticancer agent that is comprised of tegafur, which is a 5-fluorouracil (5-FU) prodrug, gimeracil, and oteracil potassium [5]. This drug has been used as first-line chemotherapy for gastric, colorectal, head and neck, and lung cancers in Japan, and it has recently been approved in other regions of the world for the treatment of advanced gastric cancer [6].

Oral squamous cell carcinoma (OSCC) is the most frequent malignancy that occurs in the oral region. Surgery is the core treatment procedure for resectable OSCCs, and chemotherapy and/or radiotherapy are selected as supportive treatments, depending on the characteristics of the disease. Neoadjuvant chemotherapy is also used to treat OSCCs, particularly in cases who require tissue-conserving surgery. Given its comparatively good efficacy and ease of administration, administering S-1 preoperatively is a promising approach to the management of OSCCs. Since an established prediction tool for the effectiveness of S-1 is not yet available, we aimed to develop prediction models for the sensitivity of primary OSCC cases to the preoperative administration of S-1.

2. Materials and methods

2.1. Ethics

This clinical study was screened and approved by the institutional review board (Approval no. G21044, Chiba University). In the present study, the subjects gave informed consent and the patient anonymity was preserved.

2.2. Patients

This was a multicenter clinical trial that involved the participation of 15 universities from January 2010 to December 2011. Patients diagnosed with primary OSCCs that occurred in the head and neck region participated in the study. S-1 (80–120 mg/body/day) was administered orally for 14 days. Cases were excluded if they received reduced doses of the medication or if they received the medication for shorter durations. The data from a total of 95 cases were eligible and were analyzed in this study.

2.3. Responses to S-1 administration

In the present study, objective tumor response was the primary endpoint. Thus, the sizes of the primary tumorous lesions, excluding the nodal lesions, were used for the evaluations. The response evaluation criteria in solid tumours (RECIST) version 1.1 [7] were used in the present study. Using these criteria, a response to S-1 was evaluated based on a comparison of the sum of the diameters of the target lesions detected before and after the administration of S-1. The criteria define a complete response (CR) as the disappearance of all target lesions, a partial response (PR) as at least a 30% decrease in the sum of the diameters of the target lesions, progressive disease (PD) as at least a 20% increase in the sum of the diameters of the target lesions and the sum of the diameters of the target lesions must also demonstrate an absolute increase of at least 5 mm, stable disease (SD) as neither a sufficient shrinkage to qualify for a PR or a sufficient increase to qualify for PD [7]. In addition to the RECIST criteria, we utilized another criterion, namely, “responders” or “non-responders”, using the reduction rate (%), which was expressed as the value of S-1-induced changes to the sum of the diameters relative to that observed at baseline. We categorized a “responders” as a highly responsive individual who showed a reduction rate of more than 0%, and the remaining cases were categorized as “non-responders”.

2.4. Ribonucleic acid extraction

Tissue was excised from the biopsies that included the cancerous lesion and the intact structures, and it was divided into two parts. One part underwent a pathological examination, and the ribonucleic acid (RNA) was extracted from the other part. To extract the RNA, the tissues were immediately preserved in the RNAlater® RNA Stabilization Reagent (Qiagen, Hilden, Germany). The tissue samples were homogenized using TissueLyser LT (Qiagen), and the total RNA was extracted using an RNeasy Mini Kit (Qiagen). In brief, ethanol was added to the homogenized samples and the lysate was loaded onto the RNeasy silica membrane. Concentrated total RNA was eluted in water. Complementary (c) deoxyribonucleic acid (DNA) was synthesized from the total RNA using Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK) and oligo (dT) primers (Sigma Genosys, Ishikari, Japan), according to the manufacturer's protocol.

2.5. DNA microarray analysis

The RNA was amplified from 1 μg of cDNA (Amino Allyl MessageAmp™ II aRNA Amplification Kits, Life Technologies, Carlsbad, CA, USA), and it was labeled using a one-color method and fragmented. The fragmented amplified RNA that was labeled with the fluorescent dye was hybridized with a human gene chip containing about 25,392 probe sets (3D-Gene Human Oligo Chip 25K; Toray Industries Inc., Tokyo, Japan). The fluorescence intensities were scanned using a 3D-Gene™ Scanner (Toray), and the spots were detected and analyzed using GenePix® Pro 6.1 Microarray Acquisition and Analysis Software (Molecular Devices, LLC, Sunnyvale, CA, USA).

2.6. Statistical analyses

Associations between the clinicopathological factors and the responses to S-1 were evaluated using the chi-square test, and paired and unpaired Student's t-tests. Prediction models for the sensitivity of OSCC cases to preoperative S-1 administration were developed using the microarray data and the clinical data that showed responses to S-1. The procedure used for the analyses is summarized in Figure 1. All 24,315 probes were examined in the microarray analysis. After background subtractions, normalizations, and noise filtering, the data were analyzed using 11,740 probes. Ninety-five of the cases were randomly divided into two groups, and 75 cases comprised the training data set (training DS) and 20 cases comprised the test DS. Leave-one-out cross-validation was adopted as an internal validation. Using Student's t-test and the fold change (FC) of gene expression, 75 genes were selected as feature genes (FG), based on the global DNA expression profiles and the RECIST criteria (Supplementary material 1). A support vector machine (SVM) was used as a classifier. The selected FGs were further refined using stepwise regression analysis, and the prediction models were developed using logistic regression analysis with the refined FGs. Prediction model 1 comprised CR + PR versus SD + PD, and prediction model 2 comprised responders versus non-responders. The models were validated using

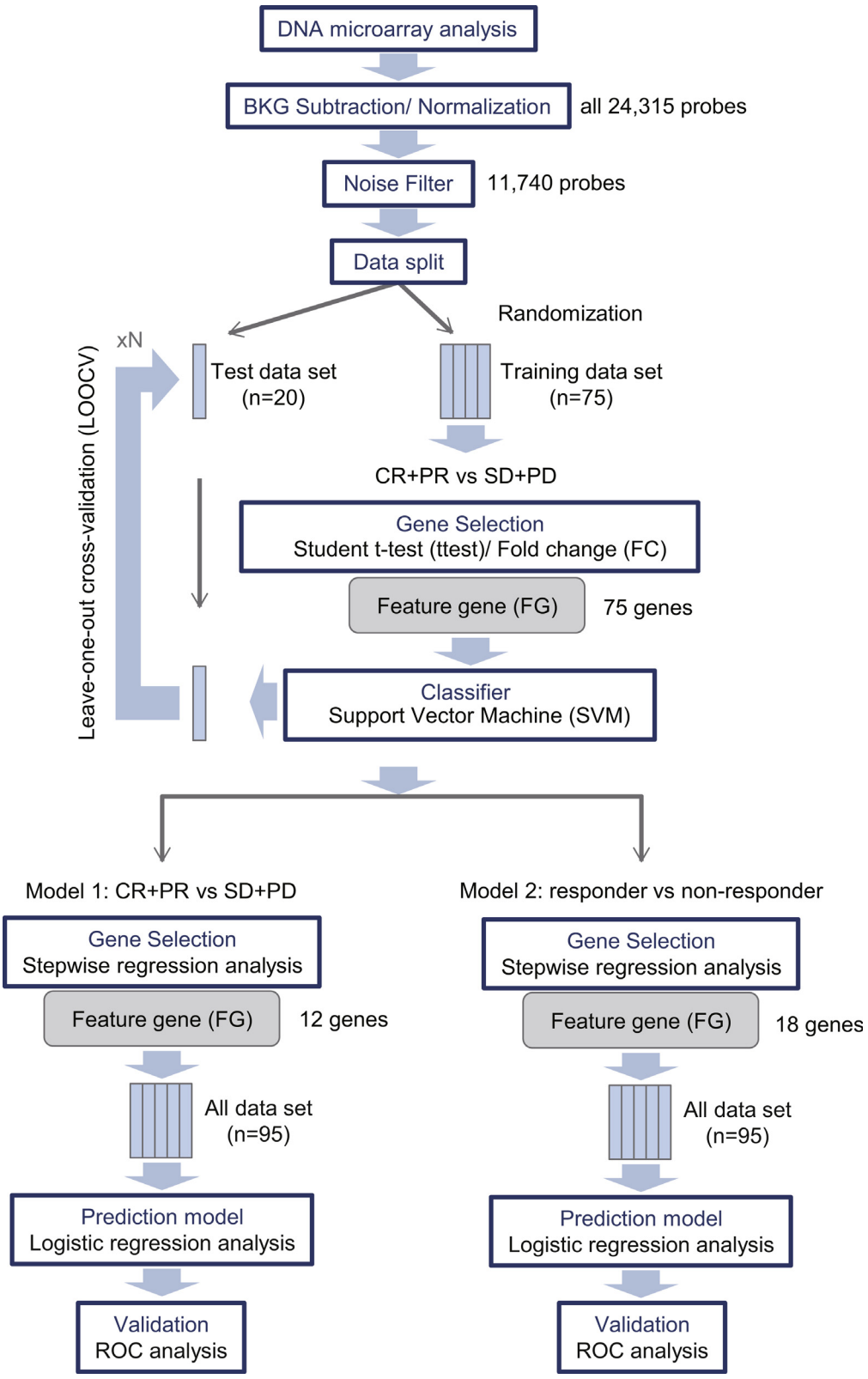

Figure 1. The procedure of prediction model development. All 24,315 probes were examined in the microarray analysis examined. Ninety-five of the cases were randomly divided into two groups, and 75 cases comprised the training data set (training DS) and 20 cases comprised the test DS. Using Student's t-test and the fold change (FC) of gene expression, 76 genes were selected as feature genes (FG). The selected FGs were further refined using stepwise regression analysis, and the prediction models were developed using logistic regression analysis with the refined FGs. Prediction model 1 comprised "CR + PR" versus "SD + PD", and prediction model 2 comprised "responders" versus "non-responders". The models were validated using receiver operating characteristic (ROC) curve analysis. P values <0.05 were considered significant.

receiver operating characteristic (ROC) curve analysis. P values <0.05 were considered significant.

3. Results

3.1. Responses to S-1 administration

The average values of the sums of the diameters before and after S-1 administration were 32.0 (standard deviation 11.5) mm and 26.3 (standard deviation 12.0) mm, respectively. The association between a reduction in the tumor's size and the administration of S-1 was significant ($P < 0.001$, Student's t-test). The difference was statistically significant ($P < 0.001$, Student's t-test). None of the cases were classified as CR, and 27 cases (28.4%) were classified as PR; thus, the response rate for S-1 was 28.4% (Table 1). Sixty-four (67.4%) and 4 (4.2%) cases were classified as SD and PD, respectively. The responder group, which showed a reduction rate of more than 0%, comprised 68 cases (71.6%), and the remaining 27 cases (28.4%) were categorized as non-responders (Table 1).

3.2. Prediction model development

Twelve genes were carefully selected for model 1 that comprised CR + PR versus SD + PD (Supplementary material 2), and 18 genes were selected for model 2 that comprised the responders versus the non-responders (Supplementary material 2). Logistic regression analysis was performed using these sets of FGs and promising prediction models were developed (Tables 2 and 3). Although the selected FGs often differed depending on the prediction model, six of the genes, namely, *fibroblast growth factor-binding protein 2 (FGFBP2)*, *melanoma-associated antigen A9 (MAGEA9)*, *S100 calcium-binding protein P (S100P)*, *transcription cofactor vestigial-like protein 1 (VGLL1)*, *HRAS-like suppressor (HRASLS)*, and *insulin growth factor-like family member 3 (IGFL3)*, were common to both prediction models.

Each case was placed on a plot of the predicted values (x-axis), which were calculated using the prediction models, and the response to S-1 (y-axis) (Figure 2 A, B). The data showed that the sensitivity was 0.963, the specificity was 0.912, and the accuracy was 0.926 for model 1, and that the sensitivity was 0.956, the specificity was 0.852, and the accuracy was 0.926 for model 2. The prediction models were validated using ROC analysis. The areas under the curves were 0.967 and 0.949 for model 1 and model 2, respectively (Figure 3 A, B). And the cut off value of predicted value was 0.305 and 0.511, respectively.

4. Discussion

The data from this study demonstrated a statistically significant decrease in the sum of the diameters in response to S-1 administration, which suggests that S-1 has a clinically substantial effect on OSCC, especially when it is used as a neoadjuvant antitumor agent. In contrast, there were some cases in whom the antitumor effect was negligible following the administration of S-1. Moreover, the response to S-1 was not correlated with the clinicopathological factors in the present study. These results strongly suggest that careful investigations into the indications for S-1 are important. The results from the present study did not

Table 1. Relationship between clinical factors and response to S-1 administration.

Factors	CR + PR vs SD + PD		Responder vs non-responder	
	CR + PR (n = 27)	SD + PD (n = 68)	Responder (n = 68)	Non-responder (n = 27)
Gender				
Male	17	38	40	15
Female	10	30	28	12
Age				
Average (standard deviation)	68.6 (11.2)	66.8 (12.7)	67.3 (12.5)	67.5 (11.9)
TNM classification				
T1	5	7	9	3
T2	15	38	38	15
T3	4	7	8	3
T4	3	16	13	6
N0	21	53	54	20
N1	6	5	10	1
N2	0	9	3	6
N3	0	1	1	0
M0	26	67	67	26
M1	1	1	1	1
Stage I	5	6	8	3
Stage II	11	32	31	12
Stage III	7	8	13	2
Stage IV	4	22	16	10
Differentiation				
Well	18	48	48	18
Moderately	8	18	18	8
Poorly	1	2	2	1
Lesion site				
Tongue	15	31	37	9
Gingiva (lower)	3	15	8	10
Buccal mucosa	3	10	11	2
Gingiva (upper)	2	10	8	4
Oral floor	4	2	4	2

Table 2. Logistic regression analysis for response to S-1 administration between CR + PR and SD + PD.

					95% confidential interval	
	Coefficient	Std. Error	Chi-square	P-value	Lower	Upper
CR + PR: constant	-26.252	12.014	4.775	0.0289	2.3e-22	0.067
FGFBP2	4.054	1.324	9.357	0.0022	4.300	771.686
HRASLS	2.358	0.757	9.699	0.0018	2.396	46.615
NGB	2.001	0.783	6.534	0.0106	1.595	34.305
ANXA3	1.309	0.690	3.601	0.0578	0.958	14.306
NEFL	1.124	0.404	7.737	0.0054	1.394	6.796
S100P	0.872	0.368	5.628	0.0177	1.164	4.918
VGLL1	-1.124	0.468	5.781	0.0162	0.130	0.812
MAGA9_HUMAN	-1.251	0.504	6.171	0.0130	0.107	0.768
KLK4	-1.327	0.676	3.851	0.0497	0.070	0.998
IGFL3	-1.360	0.520	6.838	0.0089	0.093	0.711
FAM83A	-1.925	0.752	6.546	0.0105	0.033	0.638
NP_060712.2	-2.084	0.836	6.212	0.0127	0.024	0.641

Table 3. Logistic regression analysis for response to S-1 administration between responder and non-responder.

					95% confidential interval	
	Coefficient	Std. Error	Chi-square	P-value	Lower	Upper
Responder: constant	-31.550	10669	8.744	0.0031	1.6e-23	2.4e-5
C10orf30	4.105	1.319	9.680	0.0019	4.567	805.339
BCHE	2.827	1.123	6.334	0.0118	1.868	152.710
FGFBP2	2.627	0.886	8.781	0.0030	2.434	78.554
HBB	2.114	1.437	2.165	0.1412	0.496	138.421
IL1F9	1.527	0.578	6.976	0.0083	1.483	14.310
CG010_HUMAN	1.476	0.590	6.267	0.0123	1.378	13.894
S100P	0.859	0.402	4.562	0.0327	1.073	5.197
Q9BT26_HUMAN	0.492	0.251	3.829	0.0504	0.999	2.678
ARMCX6	0.468	0.412	1.289	0.2562	0.712	3.583
IGFL3	-0.590	0.283	4.350	0.0370	0.319	0.965
HRASLS	-1.013	0.520	3.800	0.0512	0.131	1.006
GSTM3	-1.014	0.507	3.994	0.0457	0.134	0.981
VGLL1	-1.040	0.422	6.071	0.0137	0.154	0.808
NP_001004332.1	-1.242	0.471	6.943	0.0084	0.115	0.727
MAGA9_HUMAN	-1.251	0.573	4.772	0.0289	0.093	0.879
SEPT3	-1.619	0.739	4.808	0.0283	0.047	0.842
OLR1	-1.659	0.635	6.822	0.0090	0.055	0.661
HBA1	-1.877	1.491	1.584	0.2082	0.008	2.847

determine any predictable clinicopathological factors. Taken together with the fact that no prediction models for S-1 have been developed to date, the precise prediction models that we have developed should be valuable for improving patients' clinical outcomes.

Although our models showed good scores using ROC analysis, AUC was 0.967 in model 1 and 0.949 in model 2, the values for specificity were slightly lower than those for sensitivity. Since false-positive results should be avoided in clinical applications, our models might require minor modifications to increase their specificities in practice. We have developed well-balanced prediction models that show high scores for accuracy. If necessary, our models can be applied using cutoff value adjustments, depending on the purpose of their applications.

Both of the prediction models we developed showed good scores when the FGs were built into the models, which suggests that appropriate combinations of the DNA expression profiles of some genes are necessary to develop precise and reliable prediction models. The selection of relevant gene combinations is likely to be important. The sets of FGs selected for the models did not completely correspond with each other, however, six FGs, namely, *FGFBP2*, *MAGEA9*, *S100P*, *VGLL1*, *HRASLS*, and *IGFL3*, were common to both prediction models. We speculate that these six genes, in particular, may play crucial roles in patients' responses to S-1, and that investigations into their biological activities should contribute to understanding chemosensitivity to S-1.

FGFBP2, which is encoded by the *FGFBP2* gene, is a 37-kD protein that is also known as killer-specific secretory protein. It is a member of the fibroblast growth factor binding protein family, and it is a serum protein that is selectively secreted by cytotoxic lymphocytes, thus, it may be involved in cytotoxic lymphocyte-mediated immunity [8]. Overexpression of the *FGFBP2* gene has been reported in malignant gliomas [9] and overexpression of the FGFBP2 protein has been described in ovarian carcinomas [10]. These studies suggest that a high level of gene and protein expression of FGFBP2 is positively correlated with survival [9, 10].

MAGEA9 is a member of the MAGEA family of proteins, and it is an oncogenic protein that functions in tumorigenesis and tumor development. The findings from previous studies suggest that its overexpression is closely associated with unfavorable survival outcomes in patients with lung, breast, laryngeal, or liver cancers [11, 12, 13, 14].

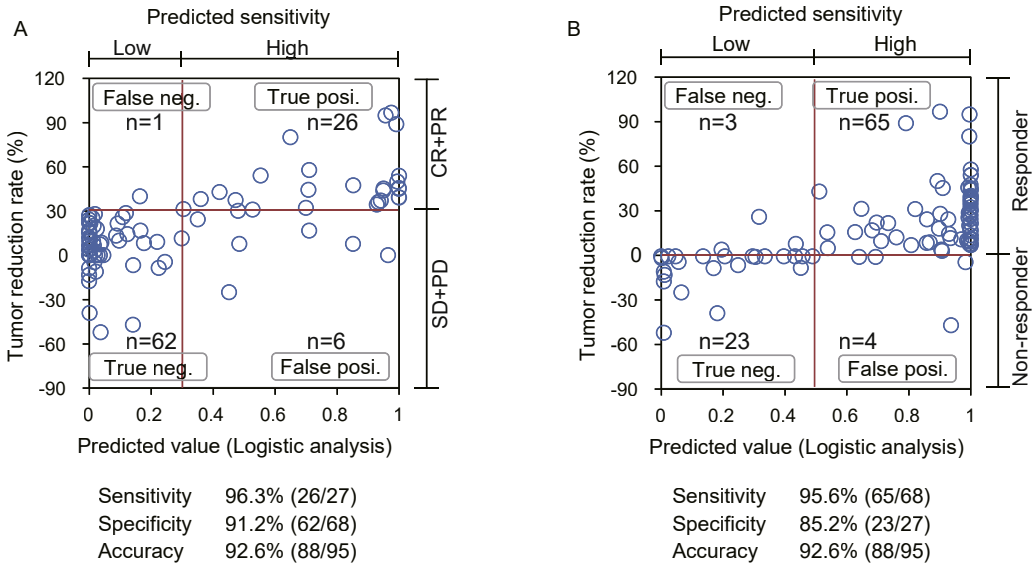

Figure 2. Distribution of cases determined by the predicted values and the response to S-1. The case distribution was of the prediction models, model 1 (A) and 2 (B), was shown in the graph. Each case was placed on a plot of the predicted values (x-axis), which were calculated using the prediction models, and the response to S-1 (y-axis). Cases were classified into 4 groups; false negative, true positive, true negative and false positive, determined by the tumor reduction rate and predicted value.

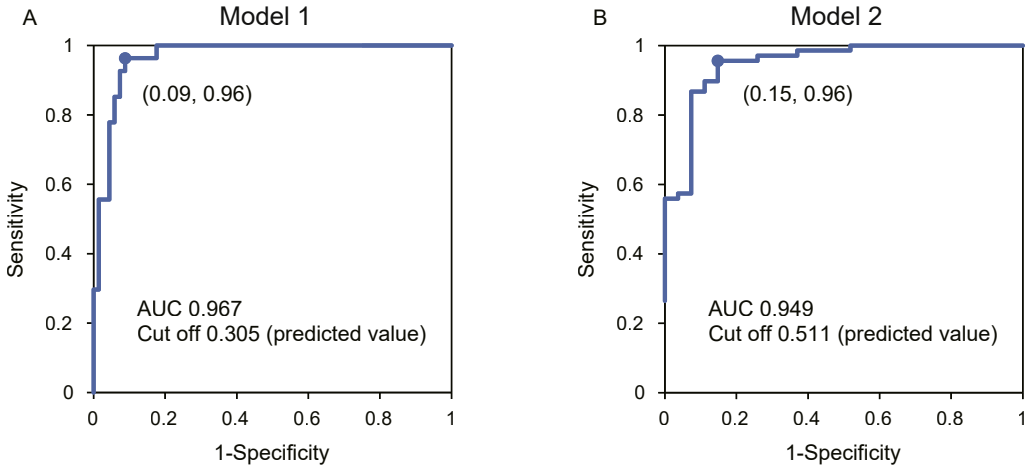

Figure 3. ROC analysis of prediction models. These models were validated using ROC analysis, and the areas under the curves (AUC) were 0.967 and 0.949 in model 1 (A) and 2 (B), respectively. The cut off of model 1 and model 2 were 0.305 and 0.511, respectively.

S100P is a member of the S100 family of proteins, and it is thought to be involved in cytoplasmic Ca^{2+} control [15]. An upregulation of S100P expression has been reported in a variety of cancers, including pancreatic and colorectal cancer [16, 17]. Furthermore, the expression of S100P has close associations with clinical staging, lymph node metastasis, and recurrence. Dong et al. suggested that a high level of S100P expression promotes cancer cell migration and invasion, and that it reduces chemosensitivity to 5-FU in vitro [16].

VGLL1 encodes a human protein that is related to the *Drosophila* transcriptional coactivator, vestigial, and is called TONDU [18]. *VGLL1* expression is associated with reduced overall survival, and it is mainly expressed in sporadic and BRCA1-associated triple negative basal-like breast carcinomas [19].

The *hrasls* gene was first cloned from mouse cell lines [20], and its partial cDNA sequence that encodes the human homolog was isolated from renal cell carcinoma cells [21]. The *hrasls* gene is thought to negatively regulate the *ras* oncogene. HRASLS proteins function as phospholipid-metabolizing enzymes and they are tumor suppressor proteins [22].

IGFL3 belongs to the insulin-like growth factor family of proteins, and it plays critical roles in cellular energy metabolism and in growth and development, particularly in prenatal growth [23]. No reports have been published that describe the tumor-related activities of IGFL3.

Based on the results from these previous studies, it is reasonable to speculate that *FGFBP2*, *MAGEA9*, *S100P*, and *VGLL1* may function as oncogenes, and that *HRASLS* might negatively regulate the development of OSCCs. Notably, *S100P* might be associated with chemosensitivity to S-1, because a high level of *S100P* expression reduces tumor cells' sensitivity to 5-FU in vitro [16]. However, no mutual relationships among the FGs highlighted in the present study have been suggested in the previous reports. Nakamura et al. investigated predictive biomarkers of adjuvant chemotherapy with S-1 after gastrectomy in Stage II/III gastric cancer patients and identified 147 upregulated and 192

downregulated genes in the favorable outcome group [24]. None of the FGs highlighted in the present study was included in their list of differentially expressed genes. This implies that clinical response to S-1 administration may differ according to the type of tumors. Hence, the molecular mechanism that regulates S-1 sensitivity is probably quite complex and is likely to be executed through many unknown pathways. We have developed promising prediction models in this study, however, the roles of FGs were still unclear because the present study focused on differential gene expressions. We believe that further functional investigations into these FGs should clarify crucial biological mechanisms associated with S-1 sensitivity.

In conclusion, the data from the present study led to the development of models that can reliably predict the sensitivity of patients with OSCC to the preoperative administration of S-1. The mechanism that regulates S-1 sensitivity remains unclear, however, the prediction models presented in this paper provide hope that further functional investigations into the FGs will lead to a greater understanding of drug resistance.

Declarations

Author contribution statement

M. Shiiba: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

H. Yamagami: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

T. Sudo and H. Tanzawa: Conceived and designed the experiments; Analyzed and interpreted the data.

Y. Tomokuni and D. Kashiwabara: Performed the experiments.

T. Kirita, J. Kusukawa, M. Komiya, K. Tei, Y. Kitagawa, Y. Imai, H. Kawamata, H. Bukawa, K. Satomura, H. Oki, K. Shinozuka, K. Sugihara, T. Sugiura, J. Sekine, H. Yokoe and K. Saito: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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