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RACK1, an excellent predictor for poor clinical outcome in oral squamous carcinoma, similar to Ki67

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ABSTRACT

Purpose: The aim of this study was to evaluate the significance of RACK1 in predicting outcome for patients with oral squamous cell carcinoma (OSCC) compared with Ki67.
Methods: The expression of both RACK1 and Ki67 in 130 patients with OSCC was illustrated by using immunohistochemistry assay. Multiple logistic regression and Pearson's correlation coefficient were used. Recurrence versus non-recurrence of the malignant lesions was considered as the surrogate for clinical outcome of patients.
Results: Multivariable logistic regression showed that the elevated RACK1 immunostaining was a factor having great influence on OSCC prognosis. RACK1 staining was strongly related to that of Ki67. The area under the receiver operating characteristic curve for both biomarkers in predicting recurrence was 0.72 and 0.70 respectively, indicating an excellent discrimination for RACK1 as well as for Ki67.
Conclusions: These data indicate that increased RACK1 expression is an important outcome predictor for OSCC compared with Ki67.

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for about 90% of malignant oral lesions, and is widely recognised as the most frequently occurring malignant tumour of oral structures. Proteomics, a study of the complete protein complements of the cell, is a promising approach in the identification of proteins which may be used as new targets for therapeutic intervention and as markers for early detection of cancers.[1]

In our most recent study, some differentially expressed proteins have been identified to be candidate biomarkers for OSCC by using a comparative proteomics approach, among which, RACK1 was chosen for detailed analysis, and the potentiality of RACK1 in the diagnosis and treatment of OSCC has been initially demonstrated.[2] RACK1, a 36-kDa homologue of the β subunit of G proteins, is a member of the WD-40 family of proteins characterised by highly conserved internal WD-40 repeats (Trp-Asp).[3] Due to its association with a large number of signalling proteins such as protein kinase C (PKC) and subunits,[4] Src and integrin β subunit,[5] phosphodiesterase PDE4D5,[6] STAT,[7] IGF-I receptor,[8] Epstein–Barr virus BZLF1 protein and HIV-1 Nef protein,[9,10] RACK1 has been identified as an anchoring or adaptor protein in multiple intracellular signal transduction pathways.

As to the role of RACK1 in carcinogenesis, four signalling pathways, including PKC, PDE4D5 (a cyclic AMP-specific

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phosphor-diesterase), tyrosine kinases/phosphatases and STAT, have been identified to interact with RACK1, involved in the coordination of cell growth, adhesion, movement, and division, although some contradictory results also exist in its oncogenic property.[11] To confirm the property, a series of experiments were designed in our previous study to investigate its full expression spectrum in clinical samples and its underlying mechanism. We have shown, by using immunohistochemistry assay, that elevated proliferation in OSCC cancer progression was associated with increased expression of RACK1 among normal epithelium, precancerous OLK tissue, and OSCC tissue. The relationship between RACK1 and some clinicopathological risk factors has been initially observed, suggesting its potential role in OSCC diagnosis.

Most studies have commonly recognised that proliferation-associated antigen Ki67 was one of the best known predictors for survival in patients with malignant diseases such as OSCC.[12] Analysis of the global gene-expression profile in OSCC cells identified a proliferation signature whereby these genes were able to discriminate patients with different median survival. RACK1 has been identified as being strongly related to proliferation of OSCC. Therefore, it is of significance to evaluate a predictive potential of RACK1 in prognosis as compared with Ki67.

In this study, we investigated whether increased expression of RACK1 could be a predictor for the poor clinical outcome of OSCC by using the cell proliferation antigen Ki67, a commonly used indicator for predicting the recurrence of malignant diseases,[13] as the comparative biological marker. With recurrence and non-recurrence as a surrogate for clinical outcome, we have shown that elevated expression of RACK1 is an excellent predictor for poor clinical outcome of OSCC. These robust molecular and genetic prognostic predictors may become an essential target in clinical practice.

2. Materials and methods

2.1. Patients

We evaluated formalin-fixed, paraffin-embedded tumour specimens from 130 OSCC patients who underwent radical neck dissections for the treatment of primary cancer at West China Stomatological Hospital, Sichuan University (Chengdu, China) between 2004 and 2007. The specimens were examined histologically after staining with H&E staining, and the clinicopathologic stage was determined according to the TNM classification system of the International Union against Cancer.[14] Tumours were classified by two experienced pathologists [Y. Chen and X. Zeng]. All of the patients or their relatives gave informed consent for the use of their tissue in the experimental procedures prior to their inclusion in the study. The project was approved by the Scientific and Ethical Committee of Sichuan University, China. The clinical data available from patient follow-up were obtained from the archives of Tumour Bank for Oral Cancer, Department of Oral Pathology and Oral Surgery. Data were evaluated to assess 'good' and 'poor' outcome at the time of selection (3 years after the radical neck dissection). All patients underwent routine chemotherapy. Oral and pharyngeal CT or MR imaging, the chest X-ray testing and abdomen sonography were performed in their follow-up visits. Cases with a good clinical outcome were defined as follows: there was no evidence of recurrent lesions at the time point of selection. Cases with a poor outcome were defined as follows: there was evidence of recurrent disease in oral mucosa or neck or other distant organs at the time point of selection. Sections were chosen for analysis when at least 70% of the slide surface area was invaded by tumour. The clinical characters of all the samples can be found in Table 1.

2.2. Antibodies

Mouse monoclonal Ki67 antigen clone was obtained from DAKO (Carpinteria, CA); polyclonal anti-RACK1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Immunohistochemistry

Immunohistochemical studies were conducted as described previously.[2] Briefly, sections were heated to 60 °C and dehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 at 95 °C. Sections were incubated with primary antibody diluted in 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 containing 1% ovalbumin and 1 mg/ml sodium azide for 12 h, followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labelled streptavidin for 15 min (LSAB-2 System; DAKO), and diaminobenzidine and

Table 1 – Patient characteristics of 130 OSCC specimens.

Factors		Number
Gender	Male	76
	Female	54
Age (year)	⩽30	36
	31–59	62
	⩾60	32
N (Lymph node metastasis)	N0	68
	N1N2	62
T stage	T1	32
	T2	52
	T3	25
	T4	21
Drinking	No	74
	Yes	56
Differentiation	Well	72
	Moderate	38
	Poor	20
Clinical stage	I	38
	II	30
	III	41
	IV	21
Tumour location	Tongue	55
	Bucca and palate	40
	Floor of mouth	20
	Gingival	15
Smoking	No	61
	Yes	69

hydrogen peroxide chromogen substrate plus diaminobenzidine enhancer (DAKO) for 10 min. Slides were counterstained with haematoxylin and mounted. The negative controls were incubated with immunoglobulin fraction in place of polyclonal primary antibody. Sections of formalin-fixed, paraffin-embedded human breast carcinoma samples were used as positive control for Ki67 and RACK1.

2.4. Semi-quantitative analysis

The degree of staining was evaluated independently by two pathologists. For RACK1, saturation and intensity of immunoassayed cells were evaluated over eight visual fields at a power of ×400 under a light microscope (Olympus Optical, Tokyo, Japan). In statistical analysis, with reference to Jeffrey's study and our previous study,[2,15] total staining of RACK1 was scored as the product of the staining intensity (on a scale of 0–3: negative = 0, weak = 1, moderate = 2, strong = 3) × the percentage of cells stained (positively recorded on an ordered categorical scale: 0 = zero, 1 = 1–25%, 2 = 26–50%, 3 = 51–100%), resulting in a scale of 0–9.

The Ki67 labelling index (LI) was determined by counting 500 cells and determining the percentage of cells that stained positively for Ki67. Positive controls were used with each staining run to identify problems with immunohistochemistry. Positive controls were also graded subjectively using the intensity scoring referred to above. All assays demonstrating inferior reactivity on positive controls were examined and repeated. Importantly, all assays were stained at the same time with the same reagents.

2.5. Data interpretation and analysis

Recurrence versus non-recurrence of the malignant lesions (3 years after the radical neck dissection) was considered as the surrogate for clinical outcome of the patients. A uniformly accurate measure of the time to recurrence was not available to provide survival analysis. Therefore, multiple logistic regressions were used to evaluate the ability of markers to predict which cases would recur and which would not. Nearly all the potential factors that could influence the probability of recurrence have been analysed, including gender, sex, smoking, drinking, tumour bulge (T), lymph node metastasis (N) and clinical stage. Pearson's correlation coefficients were used to determine whether two potential biomarkers (RACK1 and Ki67) were related to each other over all cases. To determine discrimination, the sensitivity and specificity of the given data were identified as well.

3. Results

3.1. Patient characteristics

Between April 2004 and December 2007, 130 OSCC patients who received radical neck dissection were included in the study. Table 1 summarises the characteristics of those patients.

3.2. RACK1 and Ki67 protein expression in recurrence and non-recurrence cases

We investigated whether increased staining of RACK1 correlated with clinical outcome compared with Ki67. Tissue slides were prepared from 130 cases with recurrence and non-recurrence. To determine RACK1 activation levels, the slides were stained with rabbit polyclonal antibodies and scored as described in Section 2. All of the cases exhibiting weak staining (score 2) were non- recurrence, whereas the majority (63.6%) of those exhibiting strong RACK1 staining (score 9) were OSCC recurrence (Table 2 and Fig. 1).

The Ki67 labelling index (LI) was determined by counting 500 cells and determining the percentage of cells that stained positively. As shown in Table 2 and Fig. 1, the majority (61%) of those exhibiting strong RACK1 (41–55% positive rate) staining were OSCC recurrence.

3.3. Multivariable logistic regression analysis about influencing factors for OSCC outcome

Recurrence versus non-recurrence of the malignant lesions was considered as the surrogate for clinical outcome of patients. Therefore, inclusion of all well-known clinicopathological factors with prognostic significance in our study would strengthen the utility of RACK1 as a prognostic predictor alone and in combination with other factors. Their odds ratios (ORs) and 95% confidence intervals (CI) in univariable logistic regression analysis were shown in Table 3.

Furthermore, in order to get a more precise combined analysis of all the factors and to control for confounding factors more effectively, some factors ($P < 0.20$) in univariable logistic regression would be included in the multivariable step-wise logistic regression model and be fixed. The results

Table 2 – RACK1 and Ki67 staining scores in recurrence and non-recurrence patients with OSCC.

Frequency (%) of patients exhibiting RACK1 staining in the indicated range				Frequency (%) of patients exhibiting Ki67 staining in the indicated range			
Staining score	*n*	Recurrence	Non-recurrence	Staining score (%)	*n*	Recurrence	Non-recurrence
2	17	0	10(100%)	5–15	15	1(6.7%)	14(93.7%)
3	16	2(12.5%)	14(87.5%)	16–25	31	7(22.6%)	24(77.4%)
4	32	10(31.3%)	22(68.7%)	26–32	34	10(29.4%)	24(70.6%)
6	32	27(84.4%)	5(15.6%)	33–40	27	19(70.4%)	8(29.6%)
9	33	21(63.6)	12(36.4%)	41–55	23	14(61%)	9(39%)

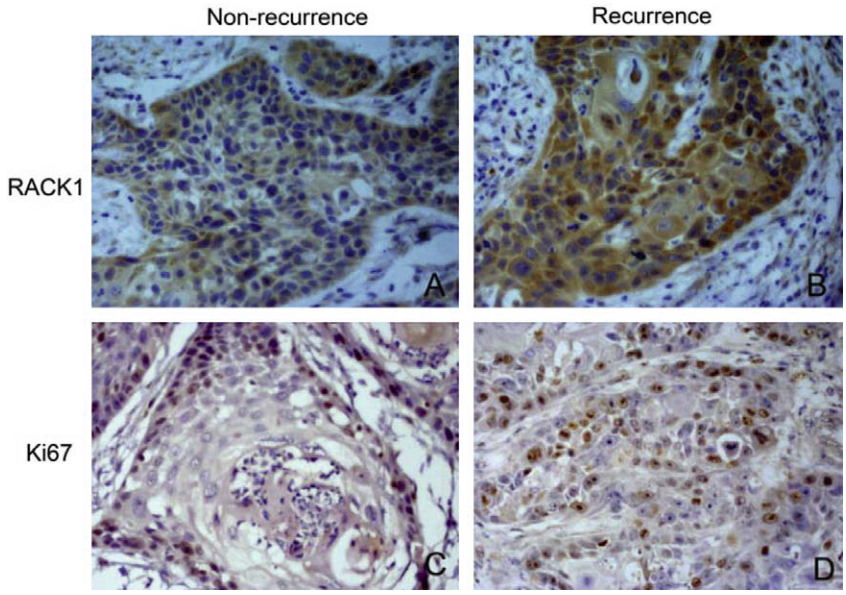

Fig. 1 – OSCC patients with recurrence (*B* and *D*) and non-recurrence (*A* and *C*). *A*: immunoperoxidase staining with RACK1 antibody of a moderately differentiated OSCC sample. Note the weak cytoplasmic staining. *B*: immunoperoxidase staining with RACK1 antibody of a well-differentiated OSCC sample. Note the strong cytoplasmic staining. *C*: immunoperoxidase staining with Ki67 antibody of the same OSCC sample as A. Note the low positive rate of nuclear staining. *D*, immunoperoxidase staining with Ki67 antibody of the OSCC sample as B. Note the high positive rate of nuclear staining (400×).

Table 3 – Univariable logistic regression analysis of influencing factors of OSCC recurrence.

Variable	β	S.E.	Wald χ^2	P value	OR[a]	95% CI[b]
Age2 versus age1	0.5175	0.3332	2.4119	0.1204	1.917	0.635–5.781
Age3 versus age1	–0.3844	0.4825	0.6348	0.4256	0.778	0.154–3.972
Gender	–0.3751	0.2345	2.5595	0.1096	0.472	0.188–1.184
Smoking	0.5675	0.2418	5.5102	0.0189	3.111	1.206–8.026
Drinking	0.4174	0.2574	2.6281	0.1050	2.304	0.840–6.321
T1 versus T4	–0.5765	0.4262	1.8292	0.1762	0.234	0.059–0.929
T2 versus T4	–1.0413	0.3915	7.0745	0.0078	0.147	0.040–0.538
T3 versus T4	0.7408	0.4745	2.4380	0.1184	0.873	0.196–3.896
N	0.3935	0.2309	2.9039	0.0884	2.197	0.889–5.431
Clinic stage	0.9053	0.2918	9.6289	0.0019	2.473	1.396–4.381
RACK1	0.8312	0.2507	10.9926	0.0009	5.271	1.973–14.083
Ki67	0.6973	0.2405	8.4080	0.0037	4.033	1.571–10.353

a OR, odds ratio.
b CI, confidence interval.

showed that smoking (OR = 4.287, 95% CI: 1.059–17.351), T2 (OR = 0.136, 95% CI: 0.018–0.629), clinic stage (OR = 3.729, 95% CI: 1.108–12.552), RACK1 (OR = 4.880, 95% CI: 1.060–22.473) and Ki67 (OR = 4.681, 95% CI: 1.008–21.744) could significantly influence the probability of recurrence (Table 4).

3.4. *Correlation between RACK1 and Ki67 expression*

The cell proliferation antigen Ki67 has often been shown to be an important predictor of poor clinical outcome with cancers. Nuclear staining for Ki67 is indicative of proliferation and is quantitated as the percentage of cells staining (Ki67 LI). The correlation between RACK1 and Ki67 expression was first evaluated. As shown in Fig. 2, the immunostaining of RACK1 was paralleled with that of Ki67 in OSCC tissues. Pearson's correlation coefficient between RACK1 and Ki67 was 0.66, $P < 0.001$ (Fig. 3).

3.5. *Elevated RACK1 alone was an excellent predictor of OSCC recurrence compared with Ki67*

To determine discrimination, the sensitivity and specificity of the given data were identified. The sensitivity of a test is defined as the true positive rate (disease present when the test is positive), whereas the specificity of a test is defined as the true negative rate (disease absent when test is negative). The area under the receiver operating characteristic (ROC) curve was determined from the plot of sensitivity *versus*

Table 4 – Multivariable logistic regression analysis of influencing factors of OSCC recurrence.

Variable	β	S.E.	Wald χ^2	P value	OR[a]	95% CI[b]
Smoking	0.7278	0.3566	4.1653	0.0413	4.287	1.059–17.351
T2 versus T4	–1.7580	0.5872	8.9615	0.0028	0.136	0.018–0.629
Clinic stage	1.3160	0.6193	4.5155	0.0336	3.729	1.108–12.552
RACK1	0.7926	0.3896	4.1394	0.0419	4.880	1.060–22.473
Ki67	0.7718	0.3918	3.8803	0.0489	4.681	1.008–21.744

a OR, odds ratio.
b CI, confidence interval.

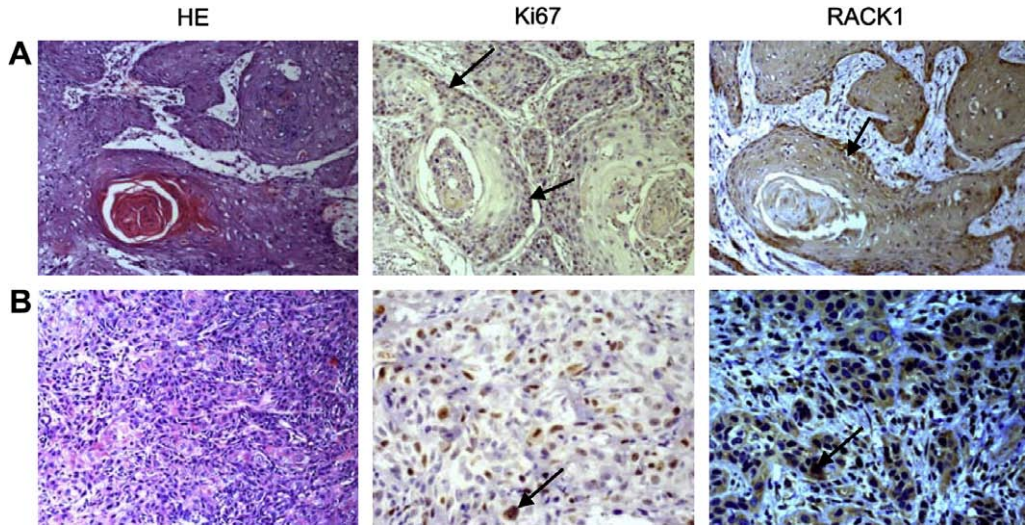

Fig. 2 – Parallel expression of Ki67 and RACK1 in human OSCC tissue. Immunohistochemical analysis showed expression of RACK1 paralleled with that of Ki67 in OSCC. The immunoreactivity of RACK1 was expressed as a product of the intensity and the proportion of cells staining positive. The positive staining of epithelium cells was expressed as yellow-brown granules with weak to moderate-strong intensity. RACK1 immunoreactivity was readily detected in the cytoplasm and occasionally in the nucleus (marked by arrow). The Ki67 labelling index (LI) was determined by the percentage of cells that stained positively in the nucleus (marked by arrow). *A*: the same visual field at a power of ×200 from a well-differentiated OSCC sample. *B*: The same visual field at a power of ×400 from a poorly-differentiated OSCC sample.

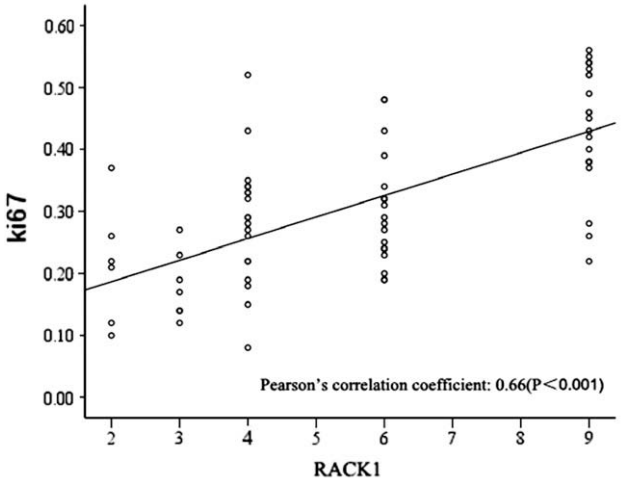

Fig. 3 – Correlation between Ki67 and RACK1. Pearson's correlation coefficient between RACK1 and Ki67 was 0.66, $P < 0.001$.

(1 – specificity) [true positive rate *versus* false positive rate] and is a measure of the predictability of a test. An area under the ROC curve of 0.7–0.9 is considered excellent discrimination, whereas a ROC value of 0.5 indicates no discrimination.[16] The ROC area for RACK1 alone was 0.72 and for Ki67 alone was 0.70, which indicates the similar value of discrimination in both of these two biomarkers (Fig. 4).

4. Discussion

Most studies have used the term biomarker to define any measurable cellular, subcellular or humoral factor that demonstrates the presence of malignant potential, or predicts tumour behaviour, prognosis or response to treatment.[17] In recent years, government, academia, industry and foundations have devoted vast resources to the identifying and developing of biomarkers, because this can help determine which treatments afford the greatest benefit to an individual, improving survival through selecting the right preventive interventions for an individual who is at high risk of tumourigenesis or poor outcome.[18]

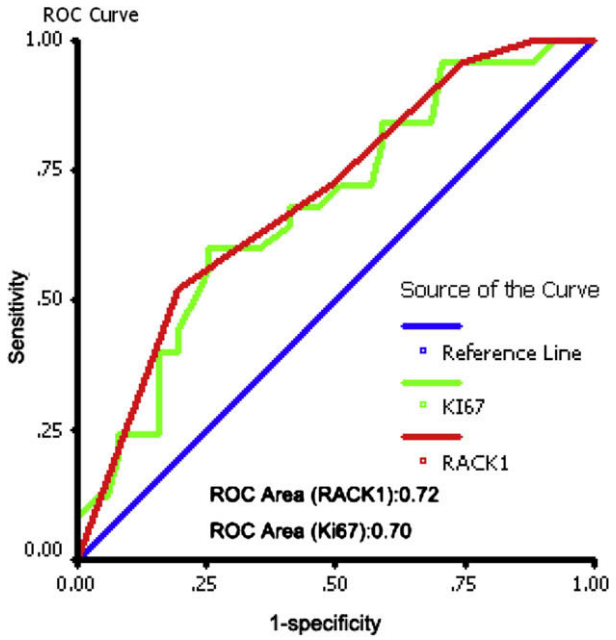

Fig. 4 – ROC curves of Ki67 and RACK1. The area under the receiver operating characteristic (ROC) curve was determined from the plot of sensitivity *versus* (1 – specificity) [true positive rate *versus* false positive rate] and is a measure of the predictability of a test. An area under the ROC curve of 0.7–0.9 is considered excellent discrimination, whereas a ROC value of 0.5 indicates no discrimination. The ROC area for RACK1 alone was 0.72, and that for Ki67 alone was 0.70.

In this study, we showed that increased RACK1 was an excellent predictor biomarker of poor clinical outcome when recurrence was used as the surrogate for poor clinical outcome. The cell proliferation antigen Ki67, which is commonly used as an indicator of proliferation, was also a good predictor of poor outcome. The expression of RACK1 was strongly related to Ki67.

Will RACK1 be a better predictor when compared with Ki67? This is a meaningful question for clinical application. In general, OSCC is believed to develop following a multi-step process, initially with pre-malignant lesions through hyperplasia to dysplasia, then carcinoma *in situ*, and finally invasive carcinoma.[19] However, the rate of proliferation alone does not determine the rate of tumour growth. In the normal epithelium, the rate of proliferation is balanced by an equal rate of apoptosis. In OSCC, there is either increased proliferation, decreased apoptosis, or both, so that the cell number increases. As noted above, four signal pathways involved in the coordination of cell growth, adhesion, movement and division have been shown to interact with RACK1. In view of this, a role of RACK1 in promoting cell proliferation seems probable. In our previous study, we demonstrated that RACK1 was a good regulator of cell survival, with significant anti-apoptotic activities, and hence would have potential to be a better predictor of aggressive oral cancer.

All those encouraging results have been generated by genomic and proteomic data. However, important issues remained unresolved concerning inconclusive or insufficient evidence to justify widespread clinical implementation, even for those biomarkers like the oestrogen receptor (ER) and ERBB2 (also known as HER2) which had been part of treatment plan for patients with ER or HER2-overexpression breast cancer.[20] Clinicians and researchers developed a set of principles and recommendations to guide the field and ensured that biomarker research resulted in clinically important applications. These were anchored to a patient-centred approach, including such guidelines so as to strengthen statistical power and adopting such standards so as to encompass methods for measurement, to collaborate and share good quality biological specimens, etc.[21,22]

As we know, this is the first time that RACK1 has been reported as an outcome predictor in oral cancer following the standard, patient-centered guidelines. Firstly, good quality clinical samples with appropriate sample sizes and grouping have been accessed. A powerful statistical method – multivariable logistic regression – has also been used. Nearly all clinicopathological indexes which were usually considered influencing factors for OSCC recurrence, including gender, age, smoking and drinking habits, lymph node involvement, tumour bulge and clinical stage, were included in the regression analysis. Compared with other studies in which only one or a few factors were included, taking all the factors together have strengthened the analysis of RACK1 as a prognostic predictor. Meanwhile, why was 'T2' the prognostically significant tumour size? It may be due to the greatest percentage of patients having tumour size T2, thus the results (T2 versus T4) were inclined to be more statistically significant.

Further, more standard biomarker assays were acquired. The high correlation of Ki67 and RACK1 has been determined. Pearson's correlation coefficient between RACK1 and Ki67 was 0.66, $P < 0.001$, suggesting their potentially similar function in carcinogenesis as proliferated-related proteins. As to the mechanism of RACK1 as an outcome predictor, some clues could be found in its correlation with lymph node metastasis which has been verified in our previous and present study. Some other work also showed RACK could improve cell adhesion, protrusion and chemotactic migration through its interaction with Src.[23] Work about whether RACK1 promotes OSCC cell invasion and adhesion using RNA interference has also been carried out.

For biomarker discovery validation studies, the sensitivity and specificity of the biomarker(s) should be provided wherever possible.[24] It is more desirable that receiver operator characteristic (ROC) curves and areas under the curves are given. In our study, RACK1 showed as good a discrimination as Ki67 when the sensitivity and specificity were identified (the ROC area for RACK1 and Ki67 was 0.72 and 0.7, respectively). Therefore, RACK1 may be a useful outcome predictor for clinically aggressive oral cancer.

In conclusion, we have shown that increased RACK1 is an important predictor of OSCC outcome. These proteins may therefore be assured as useful biological markers of clinically OSCC aggressive cancer. Replication of previous results and the inclusion of all known prognostic factors in the present study have strengthened the utility of these biomarkers alone and in combination. A replicated study that uniformly and accurately measures survival time for all the cases would also strengthen the results.

Conflict of interest statement

None declared.

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