APPENDICES

APPENDIX I

FLUORESCENT IN SITU HYBRIDIZATION ASSAY PROCEDURE (VYSIS protocol)

a. Preparing specimen slides:

1. Cut 3µm thickness of paraffin sections using a microtomes.
2. Float the sections on the distilled water bath 45°C.
3. Mount a section on the silanized slide.
4. Air dried the slides.
5. Bake the slides overnight at 36°C.

b. Deparaffinizing slides:

6. Immerse slides in Xylene I for 10 minutes at ambient temperature.
7. Immerse slides in Xylene II for 10 minutes at ambient temperature.
8. Immerse slides in Xylene III for 10 minutes at ambient temperature.
9. Dehydrate slides in 100% Ethanol I for 5 minutes at ambient temperature.
10. Dehydrate slides in 100% Ethanol II for 5 minutes at ambient temperature.
11. The slides then dried in ambient temperature for 2-5 minutes.

c. Pretreating slides:

12. Immerse slides in 0.3 HCL for 20 minutes.
13. Immerse slides in autoclaved water for 3 minutes.
14. Immerse slides in Wash Buffer I for 3 minutes.
15. Immerse slides in Pretreating solution at 80°C for 30 minutes.
16. Immerse slides in autoclaved water for 1 minute.
17. Immerse slides in Wash Buffer I for 5 minutes.
18. Immerse slides in Wash Buffer II for 5 minutes.
19. Blot excess Wash Buffer on the paper towel.

d. Enzyme Treatment:

20. Immerse slide in Protease solution (pH=2) at 37°C water bath for 80 minutes.
21. Immerse slides in Wash Buffer I for 5 minutes.
22. Immerse slides in Wash Buffer II for 5 minutes.
23. Dried slides at ambient temperature for 2-5 minutes.
24. Rehydrate slides in 70% Ethanol I for 2 minutes.
25. Rehydrate slides in 85% Ethanol II for 2 minutes.
26. Rehydrate slides in 100% Ethanol III for 2 minutes.

e. Preparing for denaturation:

1. Add the following, for each target area, to a micro centrifuge tube at room temperature.

   7µL LSI/WCP hybridization buffer.

   1 µL probe

   2 µL purified water

2. Vortex and centrifuge tube for 3 seconds.

f. Hybridization step:

27. Mark hybridization area with a diamond tipped scribe on the specimen slide.
28. Apply 10 µL of probe mixture to one target area.
29. Apply cover slip immediately.
30. Cover slip with rubber cement.
31. Place the slides in pre-warmed humidified box overnight for 12-16 hrs in 37°C incubator.

g. Post hybridization wash:

32. Preparing couplin jar with 0.4X SSC and place into a 37°C water bath for a least 30 minutes.
33. Prepare second coupling jar with 2X SSC/0.1% NP-40 at ambient temperature.
34. Take out the humidified box from the incubator, when the hybridization time is completed.
35. Remove the cover slip from each slide.
36. Immerse 4 slides each time in 0.4X SSC; agitate the slide for 1-3 seconds.
37. Leave the slides in the coupling jar for 2 minutes in 37°C water bath.
38. Immerse slides in 2X SSC in 0.1% NP-40 at ambient temperature for 2 minutes. Agitate the slides for 1-3 seconds.
39. Air dry slides in darkness at room temperature for 1 hour.

h. Counterstain with DAPI:

1. Apply 10 μL DAPI counterstain to the target area.
2. Apply cover slip immediately.
3. Seal cover slip with rubber cement.
4. Examine the fluorescent signals under Fluorescence Microscope.
5. Keep slides in darkness at -20°C freezer.

APPENDIX 2

HAEMATOXYLIN AND EOSIN STAINING METHOD

Sections were placed in different solutions as below:

1. Dewaxing sections in 2 changes of xylene for 15 minutes and 3 minutes.
2. Hydrating sections with decreasing grades of ethanol (100% - 2 minutes; 95% - 2 minutes and 70% - 2 minutes).
3. Washing sections with running tap water for 3 minutes.
4. Nuclear staining with Harris Haematoxyllin for 3 minutes.
5. Differentiating staining with acid alcohol for 2 dips.
6. Washing sections with running tap water for 3 minutes.
7. Blueing the nuclear staining with potassium acetate for 2 minutes.
8. Washing sections with running tap water for 3 minutes.
9. Hydrating with 80% ethanol for 3 dips.
10. Cytoplasmic staining with eosin for 4 minutes.
11. Dehydrating sections with increasing grades of alcohol (2 changes of ethanol 95% - 1 minute; 2 changes of ethanol 100% - 1 minute).
12. Clearing sections with xylene (3 changes of 1 minute).
13. Mounting in dibutyl phthalate in xylene (DPX).

APPENDIX 3

DEFINTION of TNM STAGING (ADOPTED FROM AJCC)

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor 2cm or less in greatest dimension
T2	Tumor more than 2cm but not more than 4cm in greatest dimension
T3	Tumor more than 4cm in greatest dimension
T4 (lip)	Tumor invades adjacent structures (e.g. through cortical bone, inferior alveolar nerve, floor of mouth and skin of face)
T4 (oral cavity)	Tumor invades adjacent structures (e.g. through cortical bone, into deep [extrinsic] muscle of tongue, maxillary sinus, skin, superficial erosion a lone of bone/tooth socket by gingival primary is not the sufficient to classify as T4)

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension

Distant Metastasis (M)

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

STAGING GROUPING

StageGroup	T Stage	N Stage	M Stage
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4	N0	M0
	T4	N1	M0
	Any T	N2	M0
IVB	Any T	N3	M0
IVC	Any T	Any N	M1

APPENDIX 4

CLINICOPATHOLOGICAL FEATURES OF OSCC OF BUCCAL MUCOSA AND TONGUE

Registeration Number:

Name:

Age:

Gender: ☐ Male ☐ Female

Race: ☐ Malay ☐ Chinese ☐ Indian

Tumour Site: ☐ Tongue ☐ Buccal Mucosa

Tumour Depth:

Histological Grading:

☐ Well-differentiated ☐ Moderately differentiated ☐ Poorly differentiated

Pathological tumor size (T): ☐ T1 ☐ T2 ☐ T3 ☐ T4

Nodal status: ☐ N0 ☐ N1 ☐ N2 ☐ N3

Pattern of invasion: ☐ Cohesive ☐ Non-Cohesive

Pathological tumor grading: ☐ Stage I ☐ Stage II ☐ Stage III ☐ Stage IV

Date of diagnosis:

Date of treatment:

Cyclin D1 amplification: ☐ Positive ☐ Negative