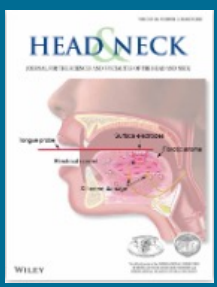
Associazione Italiana di Oncologia Cervico-Cefalica
Italian Head and Neck Oncology Society

Thirteen-gene DNA methylation analysis of oral brushing samples: A potential surveillance tool for periodic monitoring of treated patients with oral cancer



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Introduction

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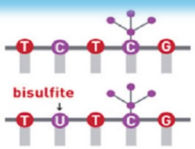
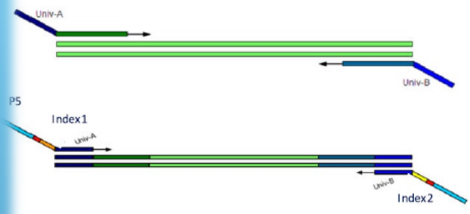
- Patients treated for Oral Squamous Cell Carcinoma (OSCC) face a 30-40% risk of developing a second neoplastic manifestation during follow-up.
- Currently, no clinically validated tests exist for early detection of recurrence in OSCC patients.
- Our research group has recently developed a non-invasive method using oral brushing and DNA methylation analysis of 13 genes to detect high-risk OSCC lesions.
- In this study, we applied this procedure to assess the association between test positivity and poor locoregional control (LRC) outcomes in patients surgically treated for OSCC.

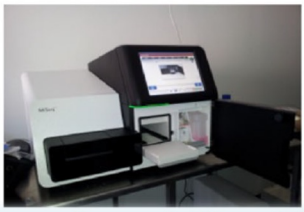


Oral brushing specimen collection
DNA purification and bisulfite treatmet



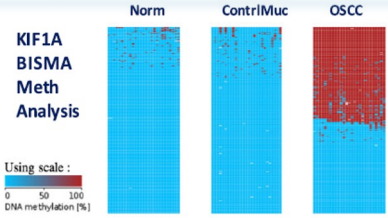
Target enrichment by multiplex PCR for the following genes:
ZAP70, ITGA4, KIF1A, PARP15, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC00599, MIR296, hTERT, GP1BB, TERC, DNMT1, H19, MIR137, PAX1, LINE1

Barcoding



Loading onto MiSEQ (Illumina) and FASTQ recruitment

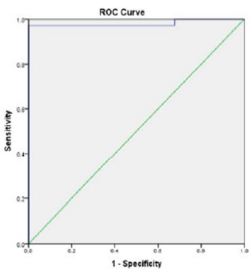
Reads filtering for Q30 and FASTA conversion
https://usegalaxy.org/



BSPAT processing

Parallel analysis by BISMA and Methylation Plotter
ROC curve analysis for each of the 355 CpGs

Selection of the most informative CpGs from *ZAP70, ITGA4, KIF1A, PARP15, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC00599, MIR296, hTERT, GP1BB*



Linear Discriminant + ROC curve analysis for the algorithm development and score calculation

Procedure Description

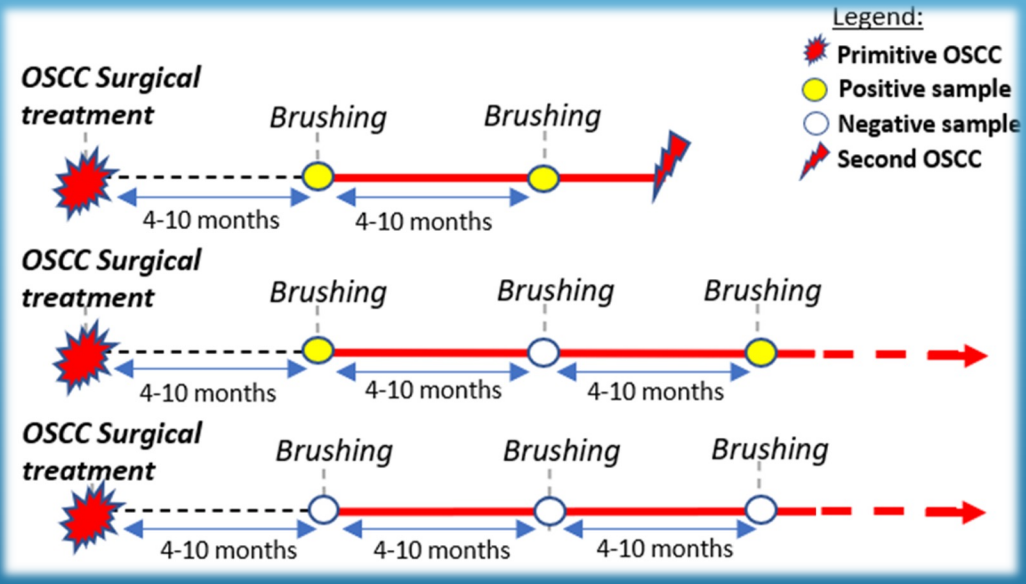
- Sample collection with Oral Brushing
- DNA extraction, bisulfite treatment, NGS-analysis & global methylation13-gene panel analysis
- Numeric value obtained through an algorithm and comparison with the cut-off to discriminate if the sample is positive or negative to the test

< -2 ____ -1.5 ____ -1 ____ 0.5 ____ 0 ____ 0.5 ____ 1.0615547 ____ 1.5 ____ >2

< 1.0615547 Negative: Normal Mucosa, Low Risk	> 1.0615547 Positive: High Risk

Materials and Methods

This is a **nested case–control study** including **61 patients** for a total **of 64 outcomes** (2/61 patients experienced multiple relapses). Each case was paired with four randomly selected controls. **Samples were collected at baseline** (4–10 months after OSCC resection**) and repeatedly every 4–10** months until relapse or death.
DNA methylation **scores were classified as persistently positive, persistently negative, or mixed.**

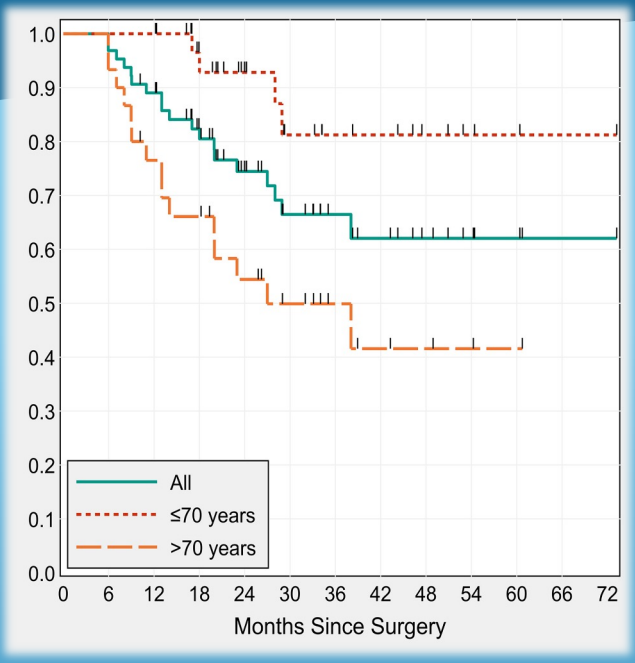


INCLUSION CRITERIA

- complete surgical resection (R0)
- no clinical or radiographic evidence of relapse within 4 months of the end of treatment
- apparently healthy mucosa without the presence of a suspected neoplastic and/or preneoplastic lesion in the regenerative area

ENDPOINT EVALUATION

Disease-free survival defined as **Time** elapsed between OSCC surgical removal and: local recurrence or second primary tumor



Kaplan–Meier survival estimates of time to relapse after surgical resection for oral squamous cell carcinoma (OSCC), overall and by age group. The spikes indicate censoring times.

Distribution of brushing score results in cases and matched controls obtained via risk-set sampling; values are count (percentage) or mean ± standard deviation.

Brushing score	Relapse cases (*n* = 19)	Matched controls (*n* = 66)[a]
Results over follow-up period		
Negative	0 (0%)	28 (42%)
Positive	15 (79%)	23 (35%)
Mixed (both neg. and pos.)	4 (21%)	15 (23%)
Last result before matching date		
Negative	1 (5%)	32 (48%)
Positive	18 (95%)	34 (52%)

• [a] Sample size is 66 instead of 19 × 4 = 76 because 76 – 66 = 10 controls had no available cytological samples between the date of surgery and the matching date, and were thus discarded

Odds ratio estimates (*p*-values) for OSCC relapse obtained with unconditional Firth-type logistic regression; the full set of pairwise comparisons between the three exposure groups is presented.

	Reference: Low		Reference: High		Reference: Mixed	
Brushing score	**Unadjusted**	**Adjusted [a]**	**Unadjusted**	**Adjusted [a]**	**Unadjusted**	**Adjusted [a]**
Negative	1.00	1.00	0.02[b]	0.04[b]	0.03[b]	0.05[b]
	(·)	(·)	(<0.001)	(<0.001)	(0.006)	(0.020)
Positive	42.15[b]	28.12[b]	1.00	1.00	1.32	1.42
	(<0.001)	(<0.001)	(·)	(·)	(0.712)	(0.639)
Mixed (neg/pos)	31.96[b]	19.75[b]	0.76	0.70	1.00	1.00
	(0.006)	(0.020)	(0.712)	(0.639)	(·)	(·)

Discussion

The present results showed that patients with **persistently positive (OR = 42) or mixed (OR = 32) scores had a significantly higher risk of OSCC relapse compared to those with persistently negative scores.**

None of the 14 patients with persistently negative scores developed a secondary tumor.

In comparison, 15 (7 Local recurrencies and 8 second primary tumors) of the 19 secondary carcinomas had persistently positive scores during follow-up

Conclusion

90% of emerging secondary neoplastic events were proceed by a positive score

It is the **first time** that a minimally-invasive tool based on DNA methylation analysis **was tested at different times as indicator of risk of relapse during oncological follow-up** program of patients treated for primary OSCC