



Feasibility of saliva testing using methylated microRNAs for head and neck squamous cell carcinoma recurrence

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Methodology

Marker discovery:

- Genome-wide search for methylation of genomic loci encoding microRNA (mgmiR)
- Quantitative methylation-specific PCR (qMS-PCR) were designed
- Candidates were screened in 16 head and neck squamous cell carcinoma (HNSCC) cell lines, tissues and saliva (Fig. 1)
- ROC curve analysis to select the best panel of 7-mgmiRs (HNKlear™) (Fig. 2)

Marker validation (retrospective study):

- qMS-PCR was performed in 281 tissues (Cancer:189, control: 92), and 194 saliva (Cancer: 86, control: 108) collected in University of Colorado and Oregon Health and Science University (Table 1).
- Univariate and multivariable logistic regression were used to derive algorithm and sensitivity and specificity were calculated in tissue and saliva (Table 2 and 3)
- Bootstrap and Random Forest analysis were used to validate using training (2/3) and testing (1/3) split and random sampling (Fig. 3)

Clinical utilities (Prospective study):

- A longitudinal prospective study for HNSCC surveillance and recurrence is ongoing. (Fig. 4)
- Kaplan-Meier survival curve was analyzed in 58 pre-treated tissues and 38 saliva from HNSCC patients (Fig. 5)
- HNKlear value was compared in HNSCC patients before and after surgery (Fig. 6)
- HNKlear test result was compared with clinical practice (imaging, biopsy) for recurrence detection (Fig. 7)

Summary of Key Findings (Retrospective Study)

- The 7-mgmiRs (9-1, 124-1, 124-2, 124-3, 129-2, 137, and 148) were identified and validated in HNSCC cell lines, tissues and saliva (Fig. 1)
- The sensitivity and specificity of the 7-mgmiRs (HNKlear) were 92.1% and 97.8% in 281 tissues (Table 2).
- The sensitivity and specificity of the 7-mgmiRs (HNKlear) plus age were 93.6% and 92.4% in 281 tissues (Table 2).
- The sensitivity and specificity of the 7-mgmiRs (HNKlear) were 84.9% and 95.4% in 194 saliva (Table 3).
- The sensitivity and specificity of the 7-mgmiRs (HNKlear) plus age were 83.7% and 95.4% in 194 saliva (Table 3).
- The internal bootstrap validation had AUC of 0.97 with a 95% CI of 0.95–0.99 in tissue and had AUC of 0.93 with a 95% CI of 0.89–0.96 in saliva
- The internal random forest validation had AUC of 0.99 with a 95% CI of 0.98–1 in tissue and had AUC of 0.94 with a 95% CI of 0.87–0.99 in saliva
- There were no difference of sensitivity for detecting oral SCC, oropharyngeal SCC, and laryngeal SCC in both tissues and saliva (Table 4).
- HNKlear detected more HPV+ HNSCCs than HPV- HNSCCs (Table 4).
- HNKlear detected 85.0% T1, 85.7% N0, or 80% stage I HNSCCs (Table 4).

Summary of Key Findings (Prospective Study)

- A longitudinal prospective study for HNSCC surveillance and recurrence is ongoing (Fig. 4)
- There were significant differences in the time to recurrence or death between the low risk and high risk groups, as displayed in the Kaplan-Meier curves for both pre-treated saliva (Fig. 5A) or tumor tissues (Fig. 5B).
- Reduction of HNKlear values upon surgery and correlation between HNKlear values of post-surgery with recurrence and/or death (Fig. 6)
- We have longitudinally followed and performed HNKlear on saliva samples of 43 SCCHN patients. Among those patients, 14 patients developed recurrence or died, HNKlear was positive in 13 of those 14 patients (92.9%); 29 patients were recurrence-free up to three years. We are continuing to follow these patients. Because patients received tests at multiple time points, we were able to detect recurrence more timely than imaging or biopsy, as shown Fig. 7, in which the test showed positive results ranging from 90-337 days before imaging and biopsy (Fig. 7).

Acknowledgement

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Retrospective Study

Fig. 1. Flow chart of mgmiRs search for HNSCC.

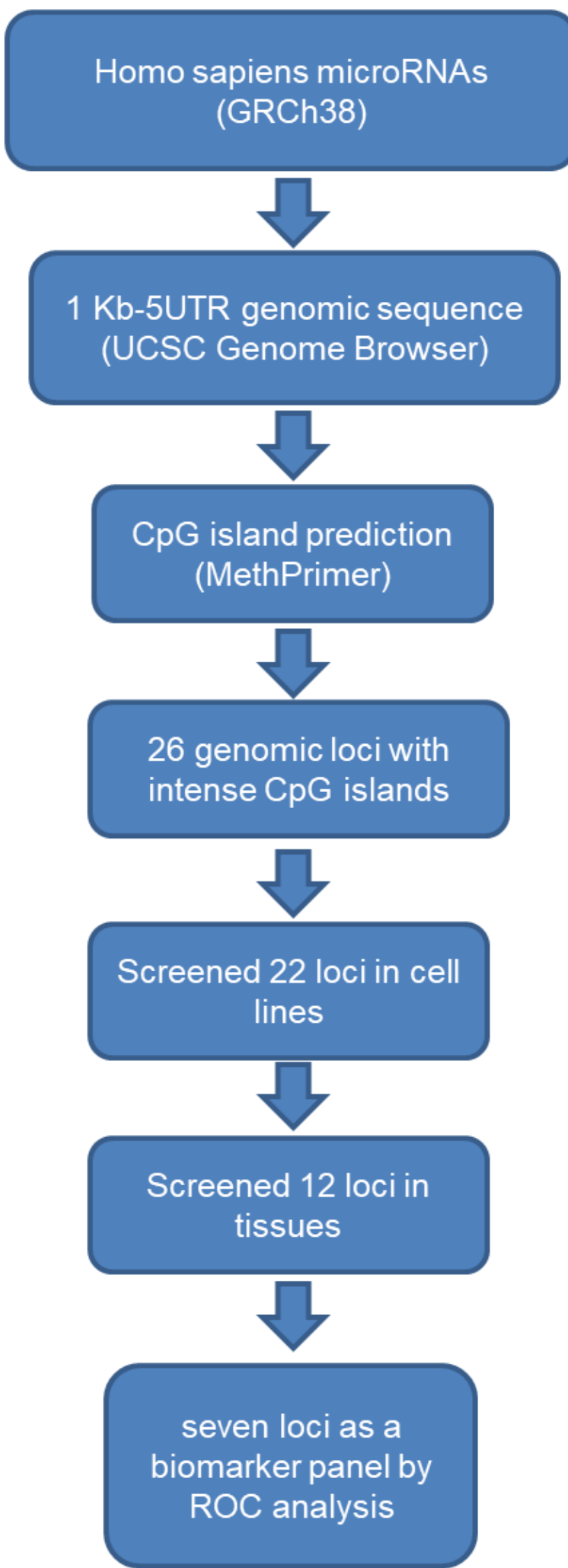


Table 1. Demographic and clinicopathological information of participated patients				
Variables	Tissue		Saliva	
	Controls (n=92)	HNSCC (n=189)	Controls (n=108)	HNSCC (n=86)
Age (y, mean±SD)	54.98±17.63	63.23±11.58*	53.77±16.04	61.07±9.66*
Gender				
Male (%)	65.2%(60/92)	70.1%(131/187)	69.4%(75/108)	77.9%(67/86)
Female (%)	34.8%(32/92)	29.9%(56/187)	30.6%(33/108)	22.1%(19/86)
Smoking status				
Never	25.6%(23/90)	22.4%(37/165)	26.9%(29/108)	19.8%(17/86)
Current	21.1%(19/90)	30.3%(50/165)	16.7%(18/108)	24.4%(21/86)
Ex-smoker	53.3%(48/90)	47.3%(78/165)	56.5%(61/108)	55.8%(48/86)
Location				
OSCC	n/a	50.8%(95/187)	n/a	50.6%(42/83)
OPSCC	n/a	23.5%(44/187)	n/a	33.7%(28/83)
LSCC	n/a	25.7%(48/187)	n/a	15.7%(13/83)
HPV status				
Positive	n/a	37.9%(25/66)	n/a	n/a
Negative	n/a	62.1%(41/66)	n/a	n/a
Tumor size				
T1	n/a	24.7%(48/186)	n/a	25.8%(20/78)
T2	n/a	26.3%(49/186)	n/a	33.3%(26/78)
T3	n/a	22.1%(41/186)	n/a	14.1%(11/78)
T4	n/a	26.9%(50/186)	n/a	26.9%(21/78)
Node status				
N0	n/a	41.9%(78/186)	n/a	36.8%(28/76)
N1	n/a	19.9%(37/186)	n/a	7.9%(6/76)
N2	n/a	35.5%(66/186)	n/a	52.8%(40/76)
N3	n/a	2.7%(5/186)	n/a	2.6%(2/76)
Stage				
I	n/a	18.3%(34/186)	n/a	13.2%(10/76)
II	n/a	10.8%(20/186)	n/a	17.1%(13/76)
III	n/a	31.7%(59/186)	n/a	22.4%(17/76)
IV	n/a	39.3%(73/186)	n/a	47.4%(36/76)

OSCC=oral SCC, OPSCC=oropharyngeal SCC, LSCC=laryngeal SCC, *p<0.05

Fig. 2. ROC curves of mgmiRs for HNSCC detection. (A). Tissue. (B). saliva.

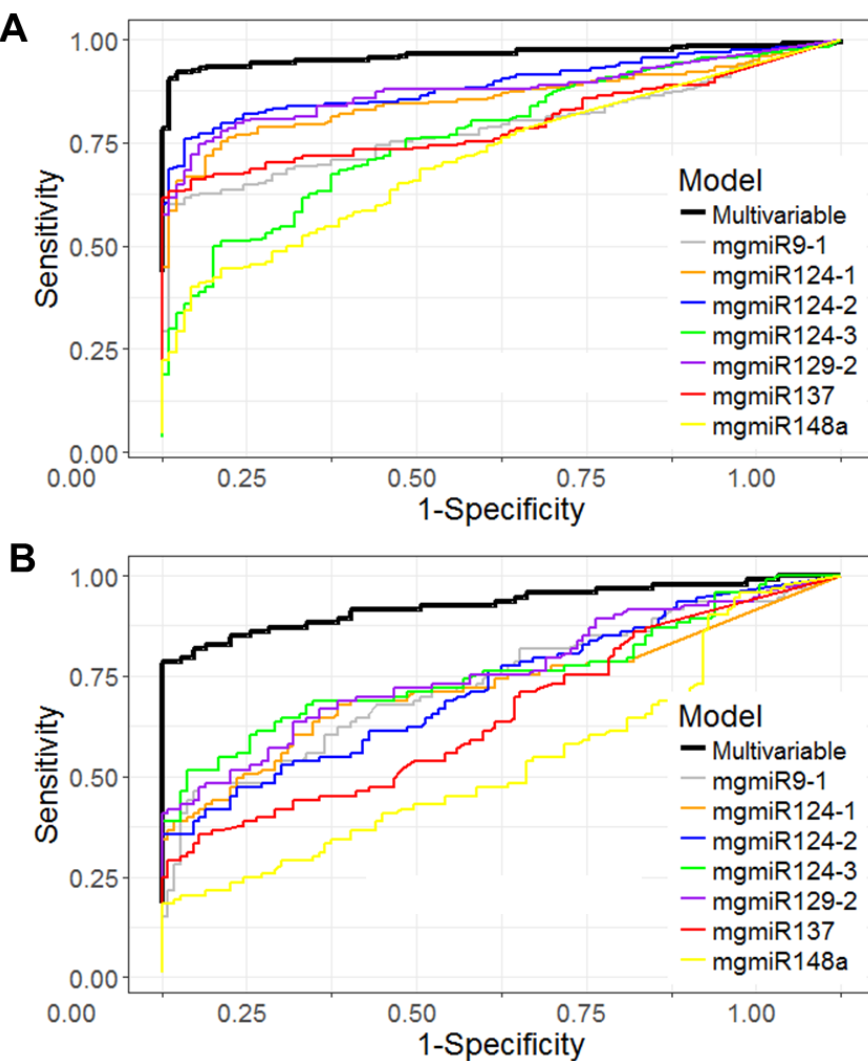


Fig. 3. Variable importance plot from the Random Forest analysis for tissue (A), or saliva

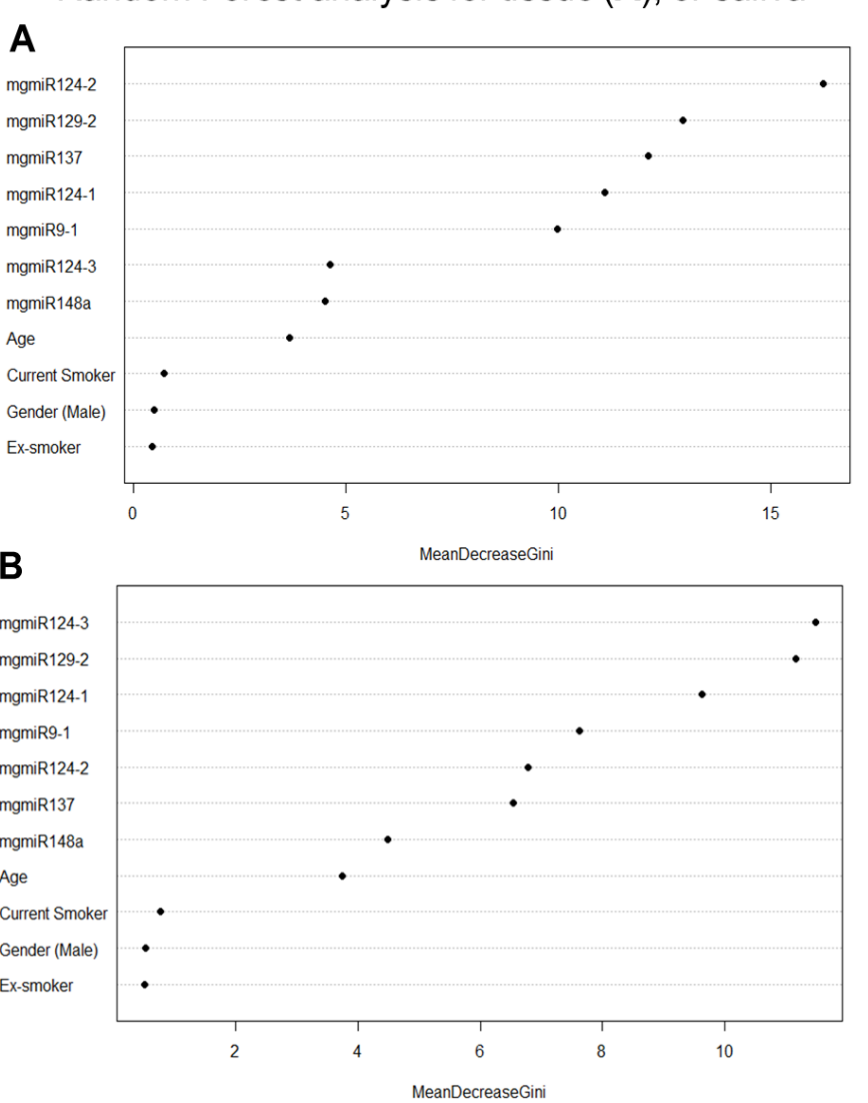


Table 2. Univariate and multivariable logistic regression in tissues			
	Sensitivity	Specificity	AUC
mgmiR9-1	75.7	65.2	0.79
mgmiR124-1	78.8	85.9	0.86
mgmiR124-2	83.1	85.9	0.90
mgmiR124-3	79.9	52.2	0.77
mgmiR129-2	80.4	87.0	0.88
mgmiR137	73.5	71.7	0.81
mgmiR148a	57.1	72.8	0.72
Combined ¹	92.1	97.8	0.96
Combined ²	93.6	92.4	0.98

Table 3. Univariate and multivariable logistic regression in saliva			
	Sensitivity	Specificity	AUC
mgmiR9-1	48.8	93.5	0.74
mgmiR124-1	66.3	70.1	0.72
mgmiR124-2	45.4	88.9	0.72
mgmiR124-3	53.5	96.3	0.76
mgmiR129-2	72.1	74.1	0.76
mgmiR137	33.7	94.4	0.66
mgmiR148a	19.8	97.2	0.54
Combined ¹	84.9	95.4	0.95
Combined ²	83.7	95.4	0.95

¹ 7 mgmiRs ¹; ² 7 mgmiRs + age

Table 4. Association between HNKlear™ and clinicopathological characteristics			
Variables	Combined mgmiRs-- -HNKlear™ % (n)	Variables	Combined mgmiRs-- -HNKlear™ % (n)
Location		Location	
OSCC (95)	92.6 (88)	OSCC (42)	83.3 (35)
OPSCC (44)	97.7 (43)	OPSCC (28)	85.7 (24)
LSCC (48)	89.8 (43)	LSCC (13)	84.6 (11)
Smoking		Smoking	
Never (37)	89.2 (33)	Never (17)	88.2 (15)
Current (50)	96.0 (48)	Current (21)	85.7 (18)
Ex (78)	96.2 (75)	Ex (48)	83.3 (36)
HPV			
Positive (25)	96.0 (24)		
Negative (41)	80.5 (33)*		
Tumor Size		Tumor Size	
T1 (46)	93.5 (43)	T1 (20)	85.0 (17)
T2 (49)	95.9 (47)	T2 (28)	84.6 (22)
T3 (41)	87.8 (36)	T3 (11)	81.8 (9)
T4 (50)	94.0 (47)	T4 (21)	85.7 (18)
Node status		Node status	
N0 (78)	94.9(74)	N0(28)	85.7 (24)
N+(108)	91.7(99)	N+(48)	83.3 (40)
Stage		Stage	
I (34)	91.2 (31)	I (10)	80.0 (8)
II (20)	95.0 (19)	II (13)	84.6 (11)
III (59)	91.5 (54)	III (17)	88.2 (15)
IV (73)	94.5 (69)	IV (36)	86.1 (31)

OSCC=oral SCC, OPSCC=oropharyngeal SCC, LSCC=laryngeal SCC, *p<0.05

Fig. 4. Standard of Care (SOC) surveillance in head and neck cancer and potential clinical utilities of HNKlear™

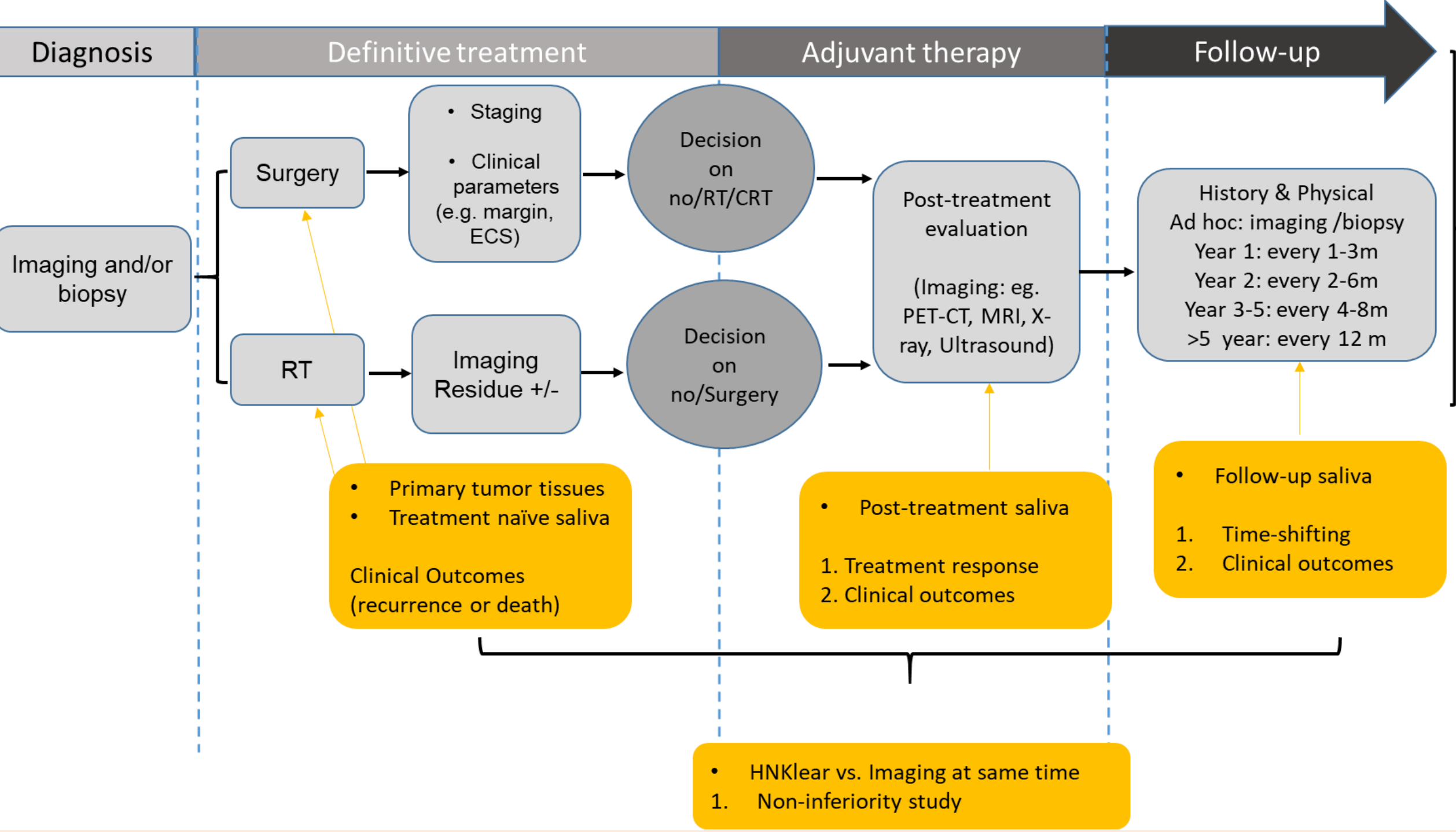


Fig. 5. Kaplan-Meier survival curve of using HNKlear from pre-treatment saliva (A), or tissue (B).

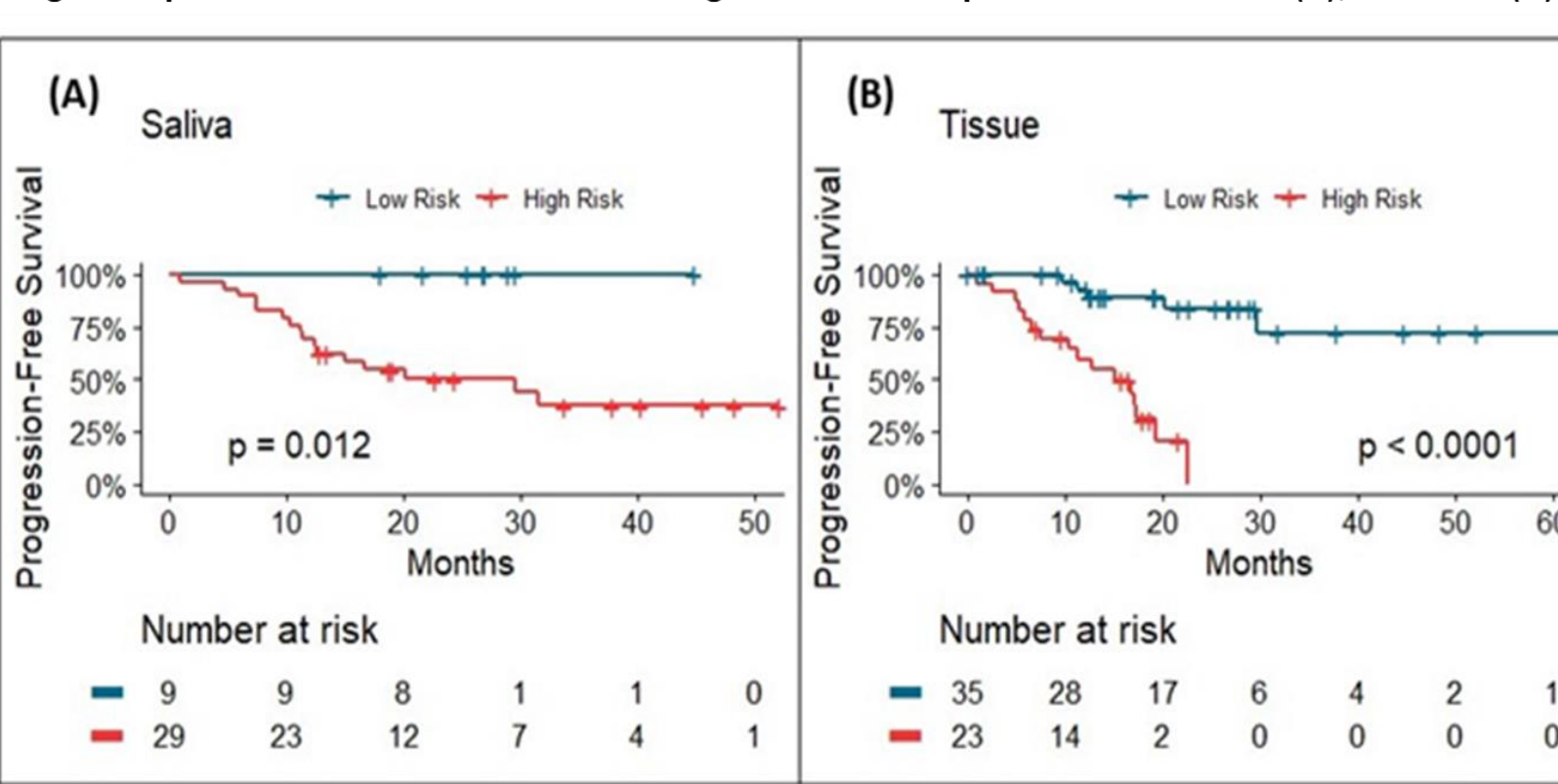


Fig. 6 Reduction of HNKlear values upon surgery and correlation between HNKlear values of post-surgery with recurrence and/or death.

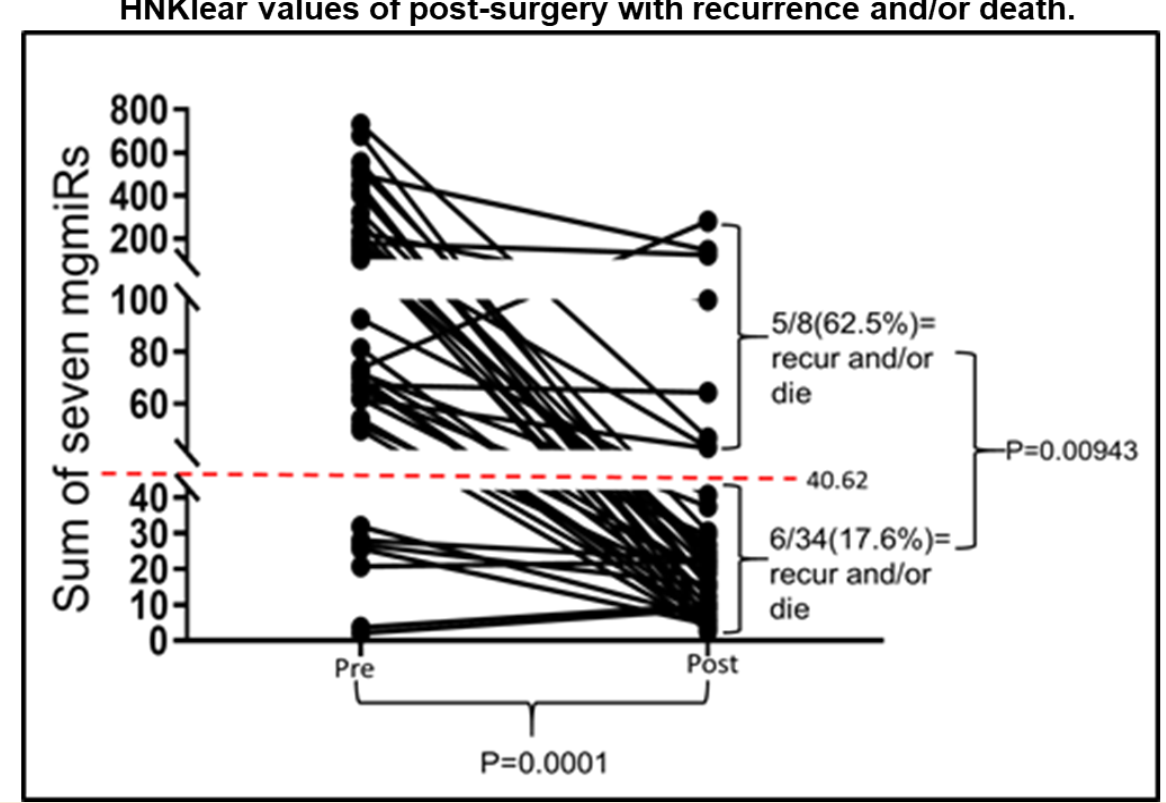


Fig. 7. Summary of HNKlear on patient follow-up and clinical outcomes in a longitudinal study on HNSCC patients.

