




# High DNMT1 Is Associated With Worse Local Control in Early-Stage Laryngeal Squamous Cell Carcinoma

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**Objectives/Hypothesis:** Early-stage laryngeal squamous cell carcinoma (LSCC) has yielded local control rates of 75% after radiotherapy. DNA methylation, in which DNA methyltransferases play an important role, has influence on tumorigenesis. In this study, we investigated the association between the expression of DNA methyltransferase 1 (DNMT1) and local control in early-stage LSCC treated with radiotherapy.

**Study Design:** Retrospective cohort study.

**Methods:** We analyzed a well-defined homogeneous series of 125 LSCC patients treated with radiotherapy with curative intent. The association of immunohistochemical expression of DNMT1 with local control was evaluated using Cox proportional hazard regression models.

**Results:** With a median follow-up of 58 months, 29 local recurrences (23%) were observed. On univariate analysis, worse local control was associated with high DNMT1 expression (hazard ratio [HR] 2.57, 95% confidence interval [CI] 1.10–6.01). Also, higher T-stage (HR 2.48, 95% CI 1.06–5.80) and positive N-status (HR 2.62, 95% CI 1.06–6.47) were associated with worse local control. Multivariate Cox regression demonstrated that high DNMT1 (HR 2.81; 95% CI 1.20–6.58) was independently associated with worse local control.

**Conclusions:** We found an association between high DNMT1 expression and worse local control in a homogeneous well-defined cohort of early-stage LSCC patients treated with definitive radiotherapy. The association between DNA methylation status as determined by DNMT1 expression and local control suggests that DNMT1 acts as a potential prognostic tumor marker in treatment decision-making in early-stage laryngeal carcinoma.

**Level of evidence:** NA

**Key Words:** Laryngeal squamous cell carcinoma, radiotherapy, local control, DNA methylation, prognostic marker.

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## INTRODUCTION

Approximately 890,000 patients are diagnosed with head and neck cancer annually of which 25% are laryngeal squamous cell carcinomas (LSCCs) worldwide.<sup>1</sup> In the Netherlands, most early-stage LSCCs are treated with radiotherapy as single modality treatment. The exception is T1a glottic LSCC without involvement of the anterior commissure for which CO<sub>2</sub> laser surgery can be an alternative treatment. In other cases, surgery

will lead to less optimal laryngeal function.<sup>2,3</sup> The local control rate for T1 to T2 laryngeal carcinoma obtained with primary radiotherapy is 75% to 90%, which is comparable with local control rates obtained with primary surgery. Given the high impact of salvage surgery in case of a recurrence, prediction of tumors that are likely to recur after primary radiotherapy may be useful in the selection of the most optimal treatment for the individual patient.

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A promising biomarker predictive for treatment outcome is DNA methylation. DNA methylation plays an important role in tumorigenesis leading to the epigenetic silencing of the expression of cancer-related genes involved in cell cycle regulation, apoptosis, and DNA repair.<sup>4-6</sup> Therefore, changes in the methylation status of these tumor-suppressor genes may contribute to carcinogenesis, influence treatment response, and promote cancer progression.<sup>7</sup> Many studies reported on specific methylation markers associated with clinical outcome in head and neck squamous cell carcinoma (HNSCC)<sup>8-11</sup> as well as in other malignancies.<sup>12,13</sup> In addition, the overall methylation status of the genome is also associated with clinical progression and tumor development.<sup>7,14,15</sup> Increased methylation of the CpG islands located near promoter regions of tumor-suppressor genes and concomitant decreased methylation of the promoter region of specific proliferation-linked genes as well as intragenic regions gradually increase tumor progression.<sup>14</sup> The DNA methyltransferase (DNMT) proteins DNMT1, DNMT3a, and DNMT3b play an important role in the methylation process by adding methyl groups to CpG dinucleotides and are involved in both de novo methylation and maintenance of methylation status of the genome.<sup>16</sup> Therefore, expression of DNMTs is considered as a regulator of methylation status and consequently to the chromosomal stability linked to overall gene expression. The expression of DNMTs was associated with DNA hypermethylation and oncogenic activation in a variety of tumors.<sup>14</sup> In several studies on HNSCC and HNSCC cell lines, DNMT1 expression was correlated with aberrant DNA methylation.<sup>17</sup> DNMT1 has also been identified to be a potential prognostic marker in HNSCC.<sup>18-22</sup> All these observations suggest a potential role for DNMT1 expression as a prognostic tumor marker in local control in HNSCC and the availability of several DNMT1-specific (clinically validated) inhibitors may provide new opportunities to improve local control.

The aim of this study was to investigate the association between the expression of DNMT1 and local control in a well-defined homogeneous cohort of patients with early-stage LSCC all treated curatively with definitive radiotherapy.

## MATERIALS AND METHODS

### Patients

We constructed an anonymous database covering 1,286 patients diagnosed with laryngeal carcinoma in the Department of Otorhinolaryngology/Head and Neck Surgery at the University Medical Center Groningen treated between 1990 and 2008. Medical records of all patients were reviewed, and all clinical, histopathological, and follow-up data of them were collected. For the present study, patients with histologically proven stage T1 or T2 LSCCs, with no distant metastases curatively treated with definitive radiotherapy only and from which formalin-fixed and paraffin-embedded pretreatment tumor material was available, were included. The selection resulted in a cohort of 125 patients with sufficient tumor tissue for immunohistochemical staining. This same cohort was reported previously in a study on the expression of DNA-repair markers pATM, pChk2, and p53 and local control<sup>23</sup> and comprised both glottic and supraglottic

LSCCs.<sup>24,25</sup> The collection of patient data and tissue samples was approved by the Medical Ethics Committee of our hospital. The privacy rights of patients were guaranteed by converting data in an anonymous database.

### Treatment

All patients were treated with definitive radiotherapy as reported previously.<sup>24,25</sup> In short, in stage T1 tumors, a total dose of 66 Gy, using 2 Gy fractions, five times weekly were given. Stage T2 tumors were generally treated with six fractions weekly to a total dose of 70 Gy within 6 weeks. In the case of elective radiotherapy to the neck nodes, a total dose of 46 Gy was given to the primary planning target volume, with an additional boost of 70 Gy to the primary tumor and pathologic lymph nodes. Patients were clinically followed every 3 months for 2 years and every 6 months up to 5 years after completing radiotherapy.

### Immunohistochemistry

Immunostaining procedures were performed as reported previously using the mouse monoclonal antibody IMG-261A against DNMT1 (Imgenex, San Diego, CA) as described.<sup>26,27</sup> In short, 4 µm sections were cut and the first and last slides were stained with hematoxylin-eosine to determine that sufficient neoplastic cells are present. The slides were deparaffinized in xylene and rehydrated in a graded alcohol series. Antigen retrieval was performed using preheated citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) and heated in a microwave oven for 15 minutes at 300 W, and endogenous peroxidase was blocked by incubating in 0.3% peroxide solution. After 60 minute incubation with the primary IMG-261A antibody (1:100 diluted in phosphate-buffered saline 1%/bovine serum albumin), the secondary antibody EnVision (Dako, Glostrup, Denmark) was applied for 30 minutes. Slides were developed with diaminobenzidine chromogen solution, followed by hematoxylin counterstaining. As positive control tissues, sections of kidney and placenta were included according to manufacturer recommendations and previous studies.<sup>27</sup> DNMT1 staining was observed in nucleus and cytoplasm. The staining was semiquantitatively scored, independently by two observers and under supervision of a head and neck pathologist. For the observers,

TABLE I.  
Patient and Tumor Characteristics of Patients (n = 125).

Characteristics	No. of Patients (%)
Age, yr	
Median (range)	65 (33-95)
Gender	
Male	107 (86)
Female	18 (14)
Sublocation	
Glottic	87 (70)
Supraglottic	38 (30)
T status	
T <sub>1</sub>	51 (41)
T <sub>2</sub>	74 (59)
N status	
N <sub>0</sub>	111 (89)
N <sub>+</sub>	14 (11)

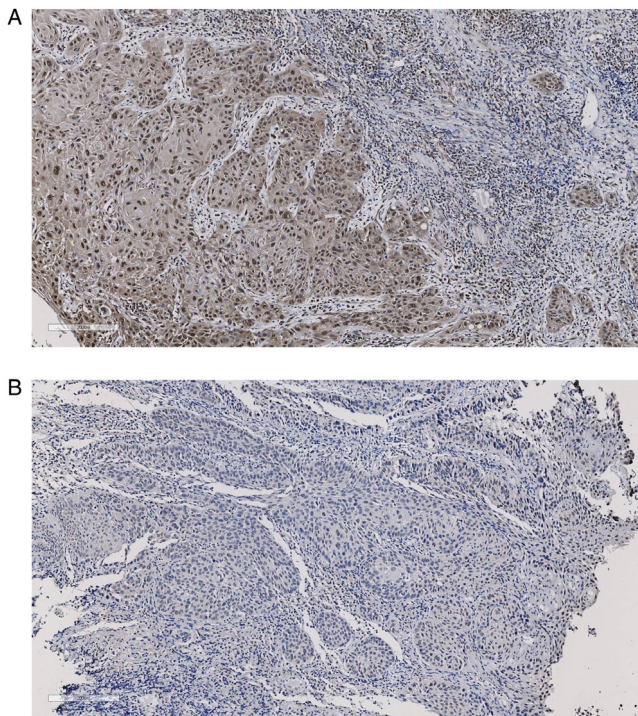


Fig. 1. Examples of laryngeal tumor biopsies showing high expression (A) and low expression (B) of DNA methyltransferase 1. Original magnification 200 $\times$ .

tissue samples were nonidentifiable. Only the administrator of the database could link tissue numbers to clinical data. All tumor cells showed cytoplasmic staining that was considered

nonspecific. The percentage of tumor nuclei with staining stronger than the cytoplasmic background staining was scored by both observers. The cases with discordant results were discussed until consensus was reached. The cutoff for high/low expressers was based on the median percentage of positive staining in all tumors. Expression of DNMT1 was considered high when  $\geq 80\%$  of the neoplastic cells showed high staining of the nucleus.

### Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 23. In all statistical analyses, a  $P$  value  $< .05$  was considered to be statistically significant.

Time calculations were performed using the date of diagnosis as starting point and the day of local recurrence or last follow-up visit as endpoint. Local recurrence was defined as reappearing tumor growth at the primary tumor site after treatment. Univariate and multivariate Cox proportional hazard models were used to assess which patient and tumor variables were independently associated with time to local recurrence. Only variables showing a significant relationship with local control in univariate analysis ( $P < .05$ ) were included in the multivariate Cox regression model. Survival curves were plotted according to the Kaplan-Meier method for illustration and were compared with log-rank test.

## RESULTS

### Patients

Expression of DNMT1 was analyzed on pre-treatment biopsies of 125 early-stage squamous cell carcinomas of the larynx. Of these patients, 18 were women and 107 were men, and their age ranged from 33 to 95 years (median age 64 years). Tumors were primary

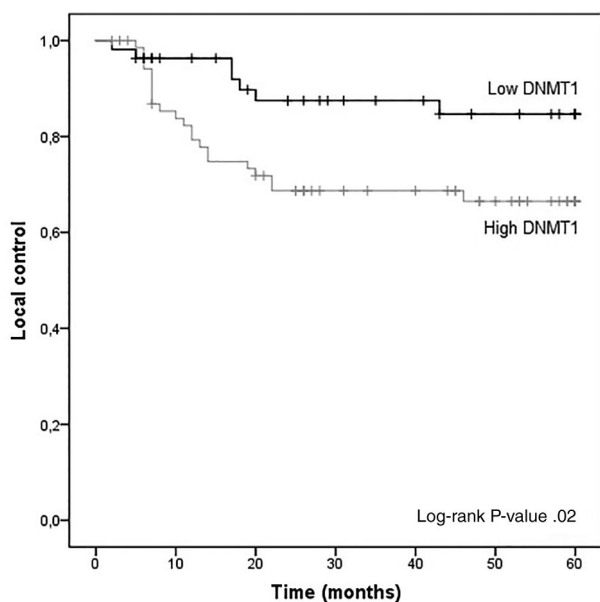
TABLE II.  
Patient Characteristics, Tumor Characteristics, and DNMT1 Expression in Relation to Local Recurrence.

	No. of Patients With Local Recurrence (%)	Univariate HR (95% CI)	$P$	Multivariate HR (95% CI)	$P$
Age					
<65	13 (21)	1			
$\geq 65$	16 (25)	1.36 (0.66–2.84)	.41		
Gender					
Male	26 (24)	1.53 (0.46–5.07)	.48		
Female	3 (17)	1			
Sublocation					
Glottic	20 (23)	1			
Supraglottic	9 (24)	1.07 (0.49–2.35)	.87		
T status					
$T_1$	7 (14)	1		1	
$T_2$	22 (30)	2.48 (1.06–5.80)	.04*	2.41 (1.00–5.80)	.05
N status					
$N_0$	23 (21)	1		1	
$N_+$	6 (43)	2.62 (1.06–6.47)	.04*	2.03 (0.80–5.12)	.14
DNMT1 expression					
Low	7 (13)	1		1	
High	22 (31)	2.57 (1.10–6.01)	.03*	2.81 (1.20–6.598)	.02*

Results of univariate and multivariate Cox regression analysis ( $n = 29$ ).

\*Signifies statistically significant relation.

CI = confidence interval; DNMT1 = DNA methyltransferase 1; HR = hazard ratio.



Low DNMT1 54 46 40 34 32 27 24  
High DNMT1 71 57 49 37 35 28 19

Fig. 2. Kaplan-Meier curve for the relation of DNA methyltransferase 1 (DNMT1) expression with local control.

localized in the glottic (70%) or supraglottic (30%) region. Table I shows patient and tumor characteristics.

### Follow-Up Data

Median follow-up was 58 months (2–60 months). In total, 29 (23%) of 125 patients developed a local recurrence within 5 years of follow-up. The median time to local recurrence was 12 months (range 2–46 months). In total, 52 patients died of which 15 died of primary disease.

### Correlation Between DNMT1 and Local Control

Almost all tumors (99%) demonstrated areas with DNMT1 expression. In 71 (56%) patients, high nuclear expression was found (see the examples of high and low expression in Figure 1).

The 5-year local control was 87% for patients with low DNMT1 expression versus 69% for patients with high DNMT1 expression. Univariate Cox regression analysis revealed that high DNMT1 expression was significantly associated with worse local control (hazard ratio [HR] 2.57, 95% confidence interval [CI] 1.10–6.01) (Table II). In addition to DNMT1 expression, higher T-stage (HR 2.48, 95% CI 1.06–5.80) and positive N-status (HR 2.62, 95% CI 1.06–6.47) were associated with worse local control. It appears that smoking is not an important parameter in our series nor have any influence on the observed DNMT1 patterns and clinical outcomes (data not shown). Multivariate Cox regression analysis (Table II) revealed that DNMT1 is an independent predictor for local control. The Kaplan-Meier survival curve illustrated that local control was significantly better in patients with low DNMT1 expression (log-rank  $P = .02$ ) (Fig. 2). Stratification of analyses

to glottic and supraglottic sublocation showed the same results, but results became insignificant most likely because pooled groups became too small (data not shown).

### DISCUSSION

In this study, we investigated the association between expression of DNMT1 and local control in a well-defined homogeneous cohort of early-stage LSCC patients treated with primary radiotherapy. We found a significant association between high DNMT1 expression and worse local control.

Different DNMT1 expression levels have been reported with aberrant DNA methylation in various malignancies and the aberrant methylation levels have been associated with clinical outcome. Interestingly, during carcinogenesis, genome-wide overall hypomethylation is observed.<sup>28</sup> This global loss of methylation contributes to tumor development through chromosomal instability as a result of changes in chromatin structure, reactivation of transposable elements such as *LINE-1*, which are normally silenced by hypermethylation, as well as loss of imprinting, which causes overexpression of genes silenced in normal tissue.<sup>14</sup> Besides genome-wide hypomethylation, CpG islands tend to become hypermethylated during tumorigenesis, which could lead to the repression of specific tumor suppression genes.<sup>14</sup> These changes are thought to be an important event in tumor progression, therapy response, invasion, and metastasis.<sup>7,10,12,14</sup> DNA methylation levels are maintained by DNMTs of which DNMT3a and DNMT3b facilitate the introduction of new DNA methylation of previously unmethylated CpG sites and DNMT1 maintains concordant DNA methylations status of opposite CpG sites on the different DNA strands.<sup>16,29</sup> Consequently, DNA methylation maintains tissue-specific DNA imprinting during cell division.<sup>30,31</sup> Therefore, changes in levels of DNMT1, considered as the regulator of the DNA methylation status of the genome, is an explanation for the observed association with clinical outcome.

Positive DNMT1 immunostaining was linked previously to lower rates of treatment response and shorter survival of patients with pharyngeal squamous cell carcinoma treated with surgery combined with adjuvant radiotherapy with/without chemotherapy or concurrent chemoradiation.<sup>20</sup> In another study, higher DNMT1 protein expression was correlated with shorter overall survival time in laryngeal carcinomas treated with surgery.<sup>21</sup> Furthermore, DNMT1 expression was positively correlated with radiation sensitization and longer survival of esophageal cancer patients.<sup>22</sup>

In our series of early-stage LSCCs, all patients showed expression of DNMT1. High expression ( $\geq 80\%$  of positive tumor cells) of DNMT1 immunostaining was found in 71 out of 125 (57%) patients, comparable to high expression levels of 47% to 75% found in oral squamous cell carcinomas.<sup>27,32</sup> One study performed immunohistochemistry with another anti-DNMT1 antibody in laryngeal carcinoma and found a percentage of 73% of positive staining ( $>10\%$  positivity in neoplastic cells was used as cutoff)<sup>21</sup> in agreement with our findings.

In our cohort, we found that high expression of DNMT1 was associated with worse local control. Inhibition of DNMT1 expression was reported to re-express several tumor suppressor genes and decreasing cell proliferation/



viability as well as inducing cell apoptosis in esophageal squamous cell carcinoma.<sup>17</sup> DNMT1 has been reported as a molecular target in a multimodality-resistant phenotype in tumor cells.<sup>18,19</sup> Epigenetic drugs such as DNMT1 inhibitors (azacitidine and decitabine) are commonly used in clinical treatment modalities<sup>33</sup> and have been reported to result in DNA hypomethylation.<sup>34</sup> This offers opportunities to investigate the modulation of the radiation sensitivity of tumors. For instance, DNMT1 inhibitors were demonstrated to sensitize HNSCC cell lines to irradiation.<sup>35</sup> These findings suggest that early-stage LSCC patients with tumors with high DNMT1 expression might benefit from adding DNMT1 inhibitors to radiotherapy. Thus far, no studies have been published that compare treatment combining DNMT1 inhibitors and radiation to DNMT1 levels in early-stage laryngeal carcinomas.

In the last decade, several specific single methylation markers have been associated with malignant disease and clinical outcome.<sup>8–12</sup> However, at this moment, only few methylation markers are applied in clinical diagnostics.<sup>36</sup> For example, *MGMT* gene promoter methylation status is currently a diagnostic test and prognostic biomarker in pediatric and adult patients with glioblastoma.<sup>36</sup> More recent studies suggested that sets of methylation markers in the early detection of cervical cancer<sup>37,38</sup> and even methylation array profiling in the diagnosis of central nervous system tumors<sup>39</sup> overall perform better than single methylation markers. The overall methylation status of the genome has also been associated with clinical progression in tumor development but as far as we know not with clinical outcome. Since DNMT1 expression is associated with genomic methylation patterns, our findings support that the genome-wide methylation status as determined by DNMT1 levels is a stronger prognostic marker than unique single methylation markers.

## CONCLUSION

We report on high DNMT1 expression as an independent predictor for worse local control in a homogeneous well-defined cohort of early-stage LSCC treated with primary radiotherapy. This association underlines the importance of the genome-wide DNA methylation status for radiotherapy response. As such DNMT1 expression-level assessment in pre-treatment tumor biopsies adds prognostic information, in addition to already used clinical factors as TNM stage and lymph node status, to aid treatment decision-making in early-stage laryngeal carcinomas.

## BIBLIOGRAPHY

- Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Abate D, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the global burden of disease study. *JAMA Oncol* 2019;27:1749–1768.
- Lefebvre JL. Laryngeal preservation in head and neck cancer: multidisciplinary approach. *Lancet Oncol* 2006;7:747–755.
- Chera BS, Amdur RJ, Morris CG, Kirwan JM, Mendenhall WM. T1N0 to T2N0 squamous cell carcinoma of the glottic larynx treated with definitive radiotherapy. *Int J Radiat Oncol Biol Phys* 2010;78:461–466.
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–2054.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–428.
- Zhang W, Xu J. DNA methyltransferases and their roles in tumorigenesis. *Biomark Res* 2017;5:1–17.

7. Azad N, Zahnow CA, Rudin CM, Baylin SB. The future of epigenetic therapy in solid tumours—lessons from the past. *Nat Rev Clin Oncol* 2013;10:256–266.
8. Clausen MJ, Melchers LJ, Mastik MF, et al. RAB25 expression is epigenetically downregulated in oral and oropharyngeal squamous cell carcinoma with lymph node metastasis. *Epigenetics* 2016;11:653–663.
9. Clausen MJ, Melchers LJ, Mastik MF, et al. Identification and validation of WISP1 as an epigenetic regulator of metastasis in oral squamous cell carcinoma. *Genes Chromosomes Cancer* 2016;55:45–59.
10. Melchers LJ, Clausen MJ, Mastik MF, et al. Identification of methylation markers for the prediction of nodal metastasis in oral and oropharyngeal squamous cell carcinoma. *Epigenetics* 2015;10:850–860.
11. Noordhuis MG, Kop EA, van der Vegt B, et al. Biological tumor markers associated with local control after primary radiotherapy in laryngeal cancer: a systematic review. *Clin Otolaryngol* 2020;45:486–494.
12. Roossink F, de Jong S, Wisman GB, et al. DNA hypermethylation biomarkers to predict response to cisplatin treatment, radiotherapy or chemoradiation: the present state of art. *Cell Oncol (Dordr)* 2012;35:231–241.
13. Ahuja N, Easwaran H, Baylin SB. Harnessing the potential of epigenetic therapy to target solid tumors. *J Clin Invest* 2014;124:56–63.
14. Esteller M. Epigenetics in cancer. *N Engl J Med* 2008;358:1148–1159.
15. Hoque MO, Kim MS, Ostrow KL, et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. *Cancer Res* 2008;68:2661–2670.
16. Gujar H, Weisenberger DJ, Liang G. The roles of human DNA methyltransferases and their isoforms in shaping the epigenome. *Genes (Basel)* 2019;10:172. <https://doi.org/10.3390/genes10020172>.
17. Zhao SL, Zhu ST, Hao X, Li P, Zhang ST. Effects of DNA methyltransferase 1 inhibition on esophageal squamous cell carcinoma. *Dis Esophagus* 2011;24:601–610.
18. Mishra MV, Bisht KS, Sun L, et al. DNMT1 as a molecular target in a multimodality-resistant phenotype in tumor cells. *Mol Cancer Res* 2008;6:243–249.
19. Graham JS, Kaye SB, Brown R. The promises and pitfalls of epigenetic therapies in solid tumours. *Eur J Cancer* 2009;45:1129–1136.
20. Chen CC, Chen WC, Wang WH, et al. Role of DNA methyltransferase 1 in pharyngeal cancer related to treatment resistance. *Head Neck* 2011;33:1132–1143.
21. Wang J, Xu Y, Li J, Sun X, Wang LP, Ji WY. The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation in laryngeal carcinoma. *Oral Oncol* 2012;48:541–546.
22. Wu S, Wang X, Chen JX, Chen Y. Predictive factors for the sensitivity of radiotherapy and prognosis of esophageal squamous cell carcinoma. *Int J Radiat Biol* 2014;90:407–413.
23. Bruine de Bruin L, Schuurin E, de Bock GH, et al. High pATM is associated with poor local control in supraglottic cancer treated with radiotherapy. *Laryngoscope* 2020;130:1954–1960.
24. Schrijvers ML, van der Laan BFAM, de Bock GH, et al. Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. *Int J Radiat Oncol Biol Phys* 2008;72:161–169.
25. Wachters JE, Schrijvers ML, Slagter-Menkema L, et al. Prognostic significance of HIF-1a, CA-IX, and OPN in T1-T2 laryngeal carcinoma treated with radiotherapy. *Laryngoscope* 2013;123:2154–2160.
26. Lin TS, Lee H, Chen RA, et al. An association of DNMT3b protein expression with P16INK4a promoter hypermethylation in non-smoking female lung cancer with human papillomavirus infection. *Cancer Lett* 2005;226:77–84.
27. Daniel FI, Rivero ER, Modolo F, et al. Immunohistochemical expression of DNA methyltransferases 1, 3a and 3b in oral leukoplakias and squamous cell carcinomas. *Arch Oral Biol* 2010;55:1024–1030.
28. Fraga MF, Herranz M, Espada J, et al. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. *Cancer Res* 2004;64:5527–5534.
29. Jorgensen HF, Bird A. MeCP2 and other methyl-CpG binding proteins. *Ment Retard Dev Disabil Res Rev* 2002;8:87–93.
30. Carlson LL, Page AW, Bestor TH. Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. *Genes Dev* 1992;6:2536–2541.
31. Pradhan S, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem* 1999;274:33002–33010.
32. Shiah SG, Chang LC, Tai KY, Lee GH, Wu CW, Shieh YS. The involvement of promoter methylation and DNA methyltransferase-1 in the regulation of EpCAM expression in oral squamous cell carcinoma. *Oral Oncol* 2009;45:e1–e8.
33. Erdmann A, Halby L, Fahy J, Arimondo PB. Targeting DNA methylation with small molecules: what's next? *J Med Chem* 2015;58:2569–2583.
34. Zielske SP. Epigenetic DNA methylation in radiation biology: on the field or on the sidelines? *J Cell Biochem* 2015;116:212–217.
35. De Schutter H, Kimpe M, Isebaert S, et al. A systematic assessment of radiation dose enhancement by 5-aza-2'-deoxycytidine and histone deacetylase inhibitors in head-and-neck squamous cell carcinoma. *Int J Radiat Oncol Biol Phys* 2009;73:904–912.
36. Locke WJ, Guanzone D, Ma C, et al. DNA methylation cancer biomarkers: translation to the clinic. *Front Genet* 2019;10:1150.
37. Boers A, Wang R, van Leeuwen RW, et al. Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. *Clin Epigenetics* 2016;8:29.
38. Eijssink JJ, Lendvai A, Derogowski V, et al. A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. *Int J Cancer* 2012;130:1861–1869.
39. Capper D, Jones DTW, Sill M, et al. DNA methylation-based classification of central nervous system tumours. *Nature* 2018;555:469–474.