Detailed analysis of adenosine A2a receptor (ADORA2A) and CD73 (5'-nucleotidase, ecto, NT5E) methylation and gene expression in head and neck squamous cell carcinoma patients

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ABSTRACT

Background: The adenosine A2a receptor (A2aR) and the adenosine synthesizing enzyme CD73 have recently evolved as a novel immunotherapeutic target. However, little is known about epigenetic modification of the encoding genes ADORA2A and NT5E.

Methods: In the present study, we evaluated methylation at 23 loci of ADORA2A and 17 loci of NT5E with regard to transcriptional activity, human papilloma virus (HPV) status, immune cell infiltration, and outcome in a cohort of 279 head and neck squamous carcinoma (HNSCC) patients obtained from The Cancer Genome Atlas (TCGA). Methylation and mRNA expression were generated by the Infinium HumanMethylation450 BeadChip and Illumina HiSeq 2000 RNA Sequencing Version 2 analysis (Illumina, Inc., San Diego, CA, USA). HPV status was assessed by RNA-Seq data analysis of the viral genes E6 and E7.

Results: Thirteen out of 23 ADORA2A loci and 15/17 NT5E loci were significantly correlated with mRNA levels (p < 0.05). Inverse correlations were predominately found in promoter regions, while positive correlations were more profound at intragenic loci. ADORA2A hypermethylation was significantly associated with poor overall survival (OS, p = 0.030), whereas NT5E hypomethylation was associated with decreased OS in HPV-positive tumors (p ≤ 0.024) and increased OS in HPV-negative HNSCC (p ≤ 0.029). Further, we found significant correlations between methylation and immune cell infiltrates.

Conclusion: Our data might point towards a significant role of the A2aR/CD73 axis during cancer progression in HNSCC.
of the A2aR on adaptive regulatory T cells (Tregs) is known to cause peripheral T cell depletion conveying immune tolerance.\textsuperscript{12,13} Ma \textit{et al.} detected high levels of A2aR expression in recurrent HNSCC and HNSCC tissues collected after induction chemotherapy.\textsuperscript{9} In their study, A2aR expression was associated with hypoxia, high numbers of tumor infiltrating CD8\textsuperscript{+} lymphocytes, and Tregs. The authors further demonstrated that pharmacological blockade of A2aR by its antagonist SCH-58261 reduced tumor growth, diminished the population of CD4\textsuperscript{+}FOXP3\textsuperscript{+} Tregs, and enhanced the anti-tumor response of CD8\textsuperscript{+} T cells in a mouse model for HNSCC. These results align with the research of Media-villa-Varela \textit{et al.} who showed similar effects for the blockade of A2aR in non-small cell lung cancer (NSCLC) and its cancer associated fibroblasts.\textsuperscript{14}

In addendum to the study by Ma \textit{et al.}, the aim of the present study was to investigate ADORA2A and NT5E DNA methylation in HNSCC with regard to gene transcription, HPV-status, and survival. An epigenetic regulation of these genes might be exploited for the development of predictive biomarkers to identify patients potentially benefitting from a treatment with A2aR antagonists.

Results

\textbf{ADORA2A and NT5E methylation and expression in isolated immune cells and cell lines}

In order to support the hypothesis of an epigenetic regulation of ADORA2A and NT5E (Fig. 1) via DNA methylation, we analyzed HNSCC cell lines and purified lymphocytes from healthy donors. We found significant methylation and mRNA differences in purified cell subsets (Supplemental Tables 1 and 2, Fig. 2 and Fig. 3). A2aR mRNA expression significantly correlated with methylation in CD4\textsuperscript{+} T cells at 20 of 23 analyzed loci (Table 1). A strong inverse correlation was found at transcription start sites (Fig. 1) targeted by bead 3 and 4 and in a region upstream and in close proximity to an alternative A2aR transcript (bead 16). All other significantly correlating loci showed positive correlations between mRNA expression and methylation. A significant inverse correlation at the locus targeted by bead 16 was also found in CD8\textsuperscript{+} T cells, while the region of the transcription start sites showed only a trend towards higher mRNA expression in cells with lower methylation (bead 3, \( p = 0.066 \)). Significant positive correlations were found at 9 out of 23 analyzed loci within the CD8\textsuperscript{+} T cell fraction. In monocytes, only one locus (targeted by bead 22) showed a significant correlation between methylation and mRNA expression.

The comprehensive methylation and expression analysis of NT5E in lymphocytes revealed significant positive correlations at 9 out of 17 analyzed loci in CD4\textsuperscript{+} T cells, 2/17 loci in CD8\textsuperscript{+} T cells, and 7 out of 17 loci in monocytes (Table 1). However, no significant inverse correlations were found.

The analyzed immune cells were obtained from healthy donors and might be different from peripheral leukocytes from HNSCC patients; however, the data shown here support the assumption of a regulatory mechanism of A2aR expression via DNA methylation in the CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells under investigation.

Furthermore, we found a significant inverse correlation between ADORA2A methylation and the donors’ age in monocytes at seven loci (at beads 2, 9, 10, 11, 12, 18, and 19, Supplemental Table 3). In CD4\textsuperscript{+} T cells, a significant negative correlation between age and methylation was detected at three of these same loci (beads 9, 11 and 19). An inverse correlation between ADORA2A mRNA expression and donors’ age was found in CD8\textsuperscript{+} T cells at the locus of bead 1 and a positive correlation at bead 16. Regarding the methylation of NT5E and donors’ age significant inverse correlations were found in CD4\textsuperscript{+} T cells (beads 12 and 15) and in monocytes (bead 13).

\textbf{A2aR and CD73 mRNA expression according to HPV-status in HNSCC}

For clinicopathological characteristics of HPV-positive and -negative patients included in the study see Table 2. Detailed analysis of A2aR mRNA in the patient cohort under investigation firstly revealed A2aR mRNA levels to be higher in HPV-positive patients compared to HPV-negative individuals (176.2 \( \pm \) 117.5 n.c. for HPV-positive, 99.9 \( \pm \) 82.3 n.c. for HPV-negative, \( p < 0.001 \); Table 2).

In HPV-positive patients, a significant difference in A2aR mRNA expression was detected with regard to the tumor location. Oropharyngeal and laryngeal tumors showed the highest normalized counts (213.8 n.c. and 303.5 n.c., respectively, \( p = 0.028 \), Table 2). In HPV-negative patients, normalized A2aR mRNA expression was shown to differ significantly between normal adjacent tissue (NAT) and HNSCC (59.8 \( \pm \) 26.7 n.c. for NAT; 99.9 \( \pm \) 82.3 n.c. for HNSCC; \( p = 0.020 \), respectively; Table 3). Analogously, normalized CD73 mRNA expression was higher in the HPV-negative cohort compared to the HPV-positive subgroup (494.6 \( \pm \) 718.0 n.c. for HPV-positive, 1495.8 \( \pm \) 1604.1 n.c. for HPV-negative, \( p < 0.001 \); Table 2). Furthermore, CD73 mRNA normalized counts significantly differed between normal adjacent and carcinomatous tissues in HPV-negative patients (438.6 \( \pm \) 441.2 n.c. for NAT; 1495.8 \( \pm \) 1604.1 n.c. for HNSCC; \( p < 0.001 \); Table 4). Additionally, differences between CD73 mRNA levels, depending on the tumor site, were seen in both subgroups (\( p = 0.004 \) in HPV-positive and \( p < 0.001 \) in HPV-negative patients, Table 2). Of note, higher A2aR mRNA normalized counts were observed in HPV-positive tumors, while increased CD73 mRNA levels were detected in HPV-negative patients (detailed data in Table 2).

\textbf{ADORA2A and NT5E methylation according to HPV-status in HNSCC}

The Infinium HumanMethylation450 BeadChip beads targeting the ADORA2A and NT5E gene loci were analyzed to identify a differential methylation between NAT and carcinomatous tissue. Within the ADORA2A gene, all analyzed loci with a significant methylation difference between NAT and HNSCC in HPV-positive patients also differed in NAT vs. tumor in HPV-negative HNSCC (Table 3 and Fig. 4). A similar observation was made for the analyzed loci within
NT5E with the exception of the regions targeted by beads 30 and 40 (Table 4 and Fig. 4).

For correlation of ADORA2A methylation with A2aR mRNA, CD73 mRNA expression levels and NT5E methylation see Tables 3 and 4 or Fig. 4. In brief, an inverse correlation was observed for methylation assessed by the majority of NT5E targeting beads. In contrast, methylation as assessed by ADORA2A targeting beads showed a positive correlation with A2aR expression, only sites targeted by the beads 18 and 19 presented with inverse correlations.

Figure 1. Genomic Organization of ADORA2A and NT5E. Shown are regulatory elements, CG-density, transcripts, transcription start sites (TSSs) and locations of beads (numbered consecutively according to their position) within the ADORA2A (A) and NT5E (B) gene locus. The modified illustration was exported from www.ensemble.org (Version 89.38) and is based on Genome Reference Consortium Human Build 38 patch release 10 (GRCh38.p10).
Figure 2. ADORA2A methylation and A2aR mRNA expression in leucocyte subtypes. A) Mean methylation of CD8<sup>+</sup> T, CD4<sup>+</sup> T, Treg, B cells, and monocytes as well as HPV-positive and HPV-negative cell lines at different loci within ADORA2A targeted by beads from the Infinium HumanMethylation450 BeadChip. B) A2aR mRNA expression in monocytes, B, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells. Asterisks imply a significant difference between two groups (*: p < 0.05, **: p < 0.001). C-E) Exemplarily illustrated methylation levels at three different loci within ADORA2A in monocytes, CD8<sup>+</sup> T, CD4<sup>+</sup> T, Treg, B cells, and HPV-positive and HPV-negative cell lines. Mean values and 95% confidence intervals are given (depicted in red).
Figure 3. NT5E methylation and CD73 mRNA expression in leucocyte subtypes. A) Mean methylation of CD8⁺ T, CD4⁺ T, Treg, B cells, and monocytes as well as HPV-positive and HPV-negative cell lines at different loci within NT5E targeted by beads from the Infinium HumanMethylation450 BeadChip. B) A2aR mRNA expression in monocytes, B, CD4⁺, and CD8⁺ T cells. Asterisks imply a significant difference between two groups (*: p < 0.05, **: p < 0.01). C-D) Exemplarily illustrated methylation levels at two different loci within NT5E in monocytes, CD8⁺ T, CD4⁺ T, Treg, B cells, and HPV-positive and HPV-negative cell lines. Mean values and 95% confidence intervals are given (depicted in red).
Survival analyses of patients stratified by A2aR mRNA, CD73 mRNA, ADORA2A methylation, and NT5E methylation

In order to identify molecular targets potentially relevant for HPV-driven carcinogenesis, A2aR and CD73 mRNA expression and beads targeting ADORA2A and NT5E gene loci were analyzed separately according to patients’ HPV status.

In HPV-positive HNSCC, low A2aR mRNA levels were associated with an adverse outcome (Fig. 5 and Table 5). Additionally, low methylation levels assessed by four ADORA2A targeting beads (beads 14 to 17) were associated with a longer OS in Kaplan-Meier analyses ($p = 0.006$, $p = 0.006$, $p = 0.005$, $p = 0.006$, respectively; and a likelihood ratio (LHR) = 13.41 for each bead, Table 5).

In the HPV-positive subgroup, low NT5E methylation and high CD73 mRNA levels were associated with an adverse outcome in HNSCC patients (Fig. 6 and Table 5). In the subgroup of HPV-negative HNSCC, defined by E6 and E7 RNA-Seq data, no prognostic impact could be shown for A2aR or CD73 mRNA expression. In contrast, methylation assessed by two NT5E targeting beads (beads 26 and 29, Fig. 6 and Table 5) and five ADORA2A targeting beads (beads 11, 12, 13, 15, and 17; Fig. 5) revealed prognostic impact for HPV-negative patients in Kaplan-Meier analyses.

Correlation of A2aR and CD73 mRNA and T cell activation markers

Significant correlations between mRNA expression of genes induced during T cell activation and A2aR mRNA expression could be shown in HPV-positive and HPV-negative subgroups, while only single markers were associated with CD73 mRNA expression (Table 6).

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**Table 1.** Correlations of mRNA expression and methylation in peripheral lymphocytes. Shown are correlations between mRNA expression and DNA methylation at various loci within the ADORA2A and NT5E genes targeted by Infinium HumanMethylation450 beads. Immune cell data were adopted by data sets GSE87650 and GSE71245. Significant features are shown in boldface.

| Bead No | Spearman’s $\rho$, Pearson’s $r$ | p-value |
|---------|---------------------------------|---------|

- **ADORA2A**

| Bead No | Spearman’s $\rho$, Pearson’s $r$ | p-value |
|---------|---------------------------------|---------|

- **NT5E**

| Bead No | Spearman’s $\rho$, Pearson’s $r$ | p-value |
|---------|---------------------------------|---------|
Table 2. Clinicopathological characteristics of HNSCC patients included in the study (n = 279). Shown are normalized counts of A2aR and CD73 mRNA. Significant features are shown in boldface.

| Variable   | HPV-positive | HPV-negative | p-value | p-value |
|------------|--------------|--------------|---------|---------|
| Patient number | 279 / 36 / 243 | 99.93 (82.26) | 1495.84 (1604.11) |
| Sex | Female | 76 / 4 / 72 | 127.22 (111.40) | 912.58 (92.21) | 0.052 |
| | Male | 203 / 32 / 171 | 182.36 (118.47) | 94.60 (77.36) | 1481.62 (1481.72) |
| Location | Oral cavity | 171 / 12 / 159 | 96.97 (51.28) | 9.46 (80.89) | 0.78 |
| | Oropharynx | 33 / 22 / 11 | 127.20 (125.65) | 106.49 (76.76) | 1463.38 (1079.41) |
| | Hypopharynx | 2 / 1 / 1 | 173.54 | 62.08 | 213.55 |
| | Larynx | 72 / 1 / 71 | 303.46 | 116.23 (85.78) | 1119.74 (1579.70) |
| Lip | 1 / 0 / 1 | . | 95.39 | 0.12 | 574.42 |
| Age [years] | Mean | 61.32 / 57.92 / 61.82 | 95.01 (88.29) | 105.15 (75.37) | 1380.20 (1538.94) |
| | Median | 61 / 59 / 62 | 95.01 (88.29) | 105.15 (75.37) | 1380.20 (1538.94) |
| Tumor category | pTis/1/2 | 93 / 14 / 79 | 176.03 (101.58) | 112.06 (104.84) | 0.070 |
| | pT3/4 | 152 / 11 / 141 | 99.61 (78.99) | 93.02 (70.07) | 1571.96 (1800.52) |
| | Unknown | 34 / 11 / 23 | 99.61 (78.99) | 93.02 (70.07) | 1571.96 (1800.52) |
| Nodal category | pN0 | 91 / 11 / 80 | 166.92 (127.83) | 87.61 (61.84) | 0.72 |
| | pN1 | 32 / 4 / 28 | 147.25 (78.80) | 112.06 (104.84) | 14144.3 (1225.88) |
| | pN2/3 | 90 / 7 / 83 | 99.61 (78.99) | 93.02 (70.07) | 1571.96 (1800.52) |
| | Unknown | 66 / 14 / 52 | 99.61 (78.99) | 93.02 (70.07) | 1571.96 (1800.52) |
| Grade | G1 | 23 / 1 / 22 | 65.53 | 230.95 | 0.24 |
| | G2 | 176 / 13 / 163 | 148.99 (105.04) | 81.24 (48.97) | 1160.87 (1015.39) |
| | G3 | 71 / 18 / 53 | 208.06 (125.58) | 97.25 (75.85) | 1387.77 (1448.29) |
| | G4 | 1 / 1 / 0 | 105.09 (62.22) | 112.66 (70.07) | 1571.96 (1800.52) |
| | Unknown | 8 / 3 / 5 | 105.09 (62.22) | 112.66 (70.07) | 1571.96 (1800.52) |
| Surgical margin | R0 | 220 / 18 / 202 | 157.86 (104.83) | 81.24 (48.97) | 1160.87 (1015.39) |
| | R1 | 23 / 7 / 16 | 141.17 (85.11) | 97.25 (75.85) | 1387.77 (1448.29) |
| | Unknown | 36 / 11 / 25 | 208.06 (125.58) | 112.66 (70.07) | 1571.96 (1800.52) |
| Smoking history | Negative | 52 / 10 / 42 | 210.06 (104.61) | 81.24 (48.97) | 1160.87 (1015.39) |
| | Positive | 220 / 26 / 194 | 163.22 (121.45) | 97.25 (75.85) | 1387.77 (1448.29) |

*As described in Statistical analysis, Kruskal-Wallis tests were applied to the variables Location, Nodal category and Grade since they contained more than two groups. NA – Not assessable.
Detailed analysis of Table 3. ADORA2A methylation and A2aR mRNA in HPV-positive and HPV-negative patients. Shown are differences between normal adjacent tissue (NAT) and tumor tissue and correlations between methylation and mRNA expression in tumor tissues by single Infinium HumanMethylation450 BeadChip bead resolution. Methylation data for NAT were available for 50 patients (six belonging to the HPV-positive cohort), while mRNA expression data for NAT were assessable for 20 individuals (two belonging to the HPV-positive cohort). Significant features are shown in boldface.

### HPV-positive (n = 36)

| Analyte (mRNA / bead) | Consecutive Number in Figs. 1, 2, 4 and 5 | Mean methylation NAT [%] | Mean methylation tumor [%] | p-value | Spearman’s 𝜋 | Pearson’s 𝜌 | Correlation: methylation and mRNA |
|-----------------------|------------------------------------------|---------------------------|----------------------------|---------|--------------|-------------|----------------------------------|
| ADORA2A mRNA          |                                          |                           |                            |         |              |             |                                  |
| cg02264779            | 1                                        | 45.15                     | 176.23                     | 0.07     | NA           | NA          | NAT vs Tumor
| cg25786366            | 2                                        | 35.2                      | 28.7                       | 0.11     | 0.281        | 0.10         | mRNA
| cg00108569            | 3                                        | 3.1                       | 3.4                        | 0.19     | 0.090        | 0.60         | mRNA
| cg07312552            | 4                                        | 1.9                       | 3.2                        | 0.075    | 0.206        | 0.23         | mRNA
| cg12144689            | 5                                        | 12.5                      | 11.9                       | 0.65     | 0.260        | 0.13         | mRNA
| cg14222656            | 6                                        | 4.9                       | 5.8                        | 0.79     | -0.012       | 0.94         | mRNA
| cg09247506            | 7                                        | 7.7                       | 11.7                       | 0.03     | 0.584        | <0.001       | mRNA
| cg26354221            | 8                                        | 94.2                      | 76.4                       | <0.001   | 0.437        | 0.008        | mRNA
| cg26001125            | 9                                        | 74.0                      | 43.0                       | <0.001   | 0.119        | 0.49         | mRNA
| cg15499799            | 10                                       | 83.3                      | 52.6                       | <0.001   | 0.537        | 0.001        | mRNA
| cg04250930            | 11                                       | 87.1                      | 55.2                       | <0.001   | 0.354        | 0.034        | mRNA
| cg01373166            | 12                                       | 78.3                      | 46.9                       | <0.001   | 0.104        | 0.55         | mRNA
| cg08025954            | 13                                       | 58.2                      | 31.4                       | <0.001   | 0.054        | 0.75         | mRNA
| cg23763137            | 14                                       | 62.9                      | 31.0                       | <0.001   | -0.050       | 0.77         | mRNA
| cg02237342            | 15                                       | 78.7                      | 38.1                       | <0.001   | -0.068       | 0.69         | mRNA
| cg1279312             | 16                                       | 68.0                      | 38.6                       | <0.001   | 0.010        | 0.95         | mRNA
| cg20660269            | 17                                       | 77.4                      | 41.5                       | <0.001   | 0.000        | 1.0          | mRNA
| cg04990420            | 18                                       | 76.8                      | 60.7                       | <0.001   | -0.072       | 0.68         | mRNA
| cg21949305            | 19                                       | 86.0                      | 77.1                       | 0.002    | -0.549       | 0.001        | mRNA
| cg21584430            | 21                                       | 63.5                      | 60.4                       | 0.96     | 0.198        | 0.25         | mRNA
| cg19855777            | 22                                       | 76.4                      | 65.5                       | 0.006    | 0.153        | 0.37         | mRNA
| cg12727256            | 23                                       | 52.2                      | 57.7                       | 0.35     | -0.166       | 0.33         | mRNA

*Values given here refer to normalized counts.

– Mann-Whitney U test, § – t-test, π – Pearson correlation, σ – Spearman correlation, NA – Not assessable.
Table 4. Detailed analysis of NT5E methylation and CD73 mRNA in HPV-positive and HPV-negative patients. Shown are differences between normal adjacent tissue (NAT) and tumor tissue and correlations between methylation and mRNA expression in tumor tissues by single Infinium HumanMethylation450 BeadChip bead resolution. Methylation data for NAT were available for 50 patients (six belonging to the HPV-positive cohort), while mRNA expression data for NAT were assessable for 20 individuals (two belonging to the HPV-positive cohort). Significant features are shown in boldface.

| Analyte (mRNA / bead) | Consecutive Number in Figs. 1, 3, 4 and 6 | Mean methylation NAT [%] | Mean methylation tumor [%] | p-value | Spearman’s ρs, Pearson’s r | p-value | Mean methylation NAT [%] | Mean methylation tumor [%] | p-value | Spearman’s ρs, Pearson’s r | p-value |
|----------------------|------------------------------------------|--------------------------|---------------------------|---------|---------------------------|---------|--------------------------|---------------------------|---------|---------------------------|---------|
| NT5E mRNA            |                                          |                          |                           |         |                           |         |                          |                           |         |                           |         |
| cg25262528           | 24                                       | 518.98$^a$               | 494.57$^b$                | 0.52$^c$| NA                        | NA      | 438.59$^e$               | 1495.84$^e$              | <0.001$^e$| NA                        | NA      |
| cg27039625           | 25                                       | 30.7                     | 71.9                      | <0.001$^e$| -0.331                    | 0.049$^e$| 40.3                     | 58.2                     | <0.001$^e$| -0.407                    | <0.001$^e$|
| cg17644557           | 26                                       | 27.3                     | 63.9                      | <0.001$^e$| -0.333                    | 0.048$^e$| 35.4                     | 47.8                     | <0.001$^e$| -0.425                    | <0.001$^e$|
| cg13315970           | 27                                       | 6.6                      | 58.0                      | <0.001$^e$| -0.440                    | 0.007$^e$| 26.9                     | 41.3                     | <0.001$^e$| -0.326                    | <0.001$^e$|
| cg21730993           | 28                                       | 6.0                      | 58.8                      | <0.001$^e$| -0.385                    | 0.020$^e$| 13.2                     | 24.8                     | 0.020$^e$| -0.466                    | <0.001$^e$|
| cg10663055           | 29                                       | 4.3                      | 36.5                      | <0.001$^e$| -0.453                    | 0.006$^e$| 5.0                      | 11.7                     | 0.005$^e$| -0.338                    | <0.001$^e$|
| cg17488985           | 30                                       | 6.8                      | 18.7                      | 0.036$^e$| -0.056                    | <0.001$^e$| 6.3                      | 7.2                      | 0.24$^e$| -0.260                    | <0.001$^e$|
| cg24635468           | 31                                       | 1.7                      | 13.9                      | 0.003$^e$| -0.059                    | 0.001$^e$| 1.7                      | 2.7                      | 0.004$^e$| -0.036                    | 0.58$^e$ |
| cg23157089           | 32                                       | 1.6                      | 13.8                      | 0.11$^e$| -0.053                    | 0.001$^e$| 1.6                      | 2.3                      | 0.27$^e$| -0.009                    | 0.89$^e$ |
| cg17966619           | 33                                       | 6.8                      | 22.7                      | 0.12$^e$| -0.040                    | 0.015$^e$| 6.7                      | 8.8                      | 0.43$^e$| -0.099                    | 0.13$^e$ |
| cg27297363           | 34                                       | 20.1                     | 39.5                      | 0.018$^e$| -0.047                    | 0.003$^e$| 22.3                     | 17.1                     | <0.001$^e$| -0.373                    | <0.001$^e$|
| cg09025339           | 35                                       | 42.2                     | 36.6                      | 0.39$^e$| 0.038                     | 0.83$^e$| 39.6                     | 37.2                     | 0.19$^e$| 0.236                     | <0.001$^e$|
| cg23172664           | 36                                       | 34.2                     | 41.5                      | 0.31$^e$| -0.029                    | 0.087$^e$| 37.6                     | 40.5                     | 0.22$^e$| 0.085                     | 0.19$^e$ |
| cg24702826           | 37                                       | 82.0                     | 72.3                      | 0.020$^e$| -0.052                    | 0.076$^e$| 76.6                     | 71.2                     | 0.008$^e$| 0.011                     | 0.87$^e$ |
| cg06516476           | 38                                       | 40.9                     | 40.7                      | 0.98$^e$| -0.376                    | 0.024$^e$| 41.5                     | 33.3                     | <0.001$^e$| -0.179                    | 0.005$^e$|
| cg03285617           | 39                                       | 74.4                     | 62.0                      | 0.066$^e$| 0.238                     | 0.16$^e$| 76.6                     | 64.0                     | <0.001$^e$| 0.331                     | <0.001$^e$|
| cg09988947           | 40                                       | 79.7                     | 53.0                      | 0.032$^e$| 0.422                     | 0.010$^e$| 67.6                     | 68.1                     | 0.17$^e$| 0.538                     | <0.001$^e$|

*Values given here refer to normalized counts.

$^a$ – Mann-Whitney U test, $^b$ – t-test, $^c$ – Pearson correlation, $^s$ – Spearman correlation, NA - Not assessable.
Correlation of A2aR mRNA, CD73 mRNA, ADORA2A methylation, and NT5E methylation with lymphocyte infiltrates

Since the expression of A2aR and CD73 is closely related to the adaptive immune cell activity, methylation and mRNA levels were quantified with respect to immune cell densities (B cells, CD4+ T cells, CD8+ T cells, dendritic cells, and regulatory T cells, adopted from Li et al.16) (see Supplemental Tables 4 and 5 for detailed analysis).

Discussion

Epigenetic alterations seem to be involved in key biological processes, especially in the lymphocyte compartment.17-20

De novo methylation further appears to contribute to T cell exhaustion.18 Additionally, methylation of distinct genes might add information about the prognosis of various malignancies.21-24 In the present study, differential ADORA2A and NT5E DNA methylation revealed an impact on the course of HNSCC. While A2aR and CD73 mRNA expression only served as a prognostic factor in HPV-positive subgroup analysis, methylation of ADORA2A and NT5E, determined by various Illumina 450 k beads, performed well as prognostic biomarkers in both subgroups. These findings are in line with the observation that HPV consistently alters the cancer cells’ epigenome, thereby affecting the host’s immunologic tolerance to tumor cells.25

Our data concur with recent observations by Ma et al., who reported a correlation between A2aR and FOXP3 expression in

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**Figure 4.** Results for individual BeadChip beads of ADORA2A and NT5E. Results of a detailed analysis of methylation for the genes ADORA2A (A and B) and NT5E (C and D) at single bead resolution with regard to the HPV-status are pictographically displayed (methylation differences between normal adjacent tissue (NAT) and tumor tissue, correlations between mRNA expression and methylation, and association with survival are shown, for exact values see Tables 3–5). Significant results are depicted in red. The beads are numbered according to Fig. 1.
human HNSCC tissue, indicating that A2aR may be expressed in Tregs.9 Furthermore, we found a strong correlation of A2aR mRNA expression with increased levels of genes known to be induced during T cell activation (granzymes, perforin, PD-1, CD69, interferon gamma, and interleukin 2 receptor subunit alpha) suggesting a role of A2aR in T cell activation. In our study, A2aR mRNA positively correlated with CD4 T cells, B cells, Tregs, and dendritic cells, whereas Ma et al. observed an inverse correlation with CD8 T cells in their experimental setup.9 However, due to post-transcriptional modification, mRNA expression, as analyzed in the present study, does not necessarily parallel protein expression in every single aspect. Of note, Ma et al. claimed that high A2aR expression could be detected in HNSCC tissues undergoing induction chemotherapy. The authors hypothesized that A2aR may facilitate drug resistance by altering the immunological tumor microenvironment. Their findings thus strongly emphasize the potential clinical relevance of the current study.

Since HPV persistence is known as a major prognostic factor in HNSCC, HPV-positive and HPV-negative subgroups were analyzed separately in the present study. As reported in earlier studies, HPV interferes with the tumor epigenome, leading to promoter methylation of various genes, especially immune related genes.26 HPV, as a result, induces a tolerant microenvironment allowing tumor prosperity.25 In HPV-positive HNSCC, high ADORA2A methylation and low NT5E methylation, respectively, were associated with shorter OS. The latter may imply that the outcome of patients with HPV-positive HNSCC is linked to the activation status of immune checkpoint pathways, e.g. the adenosine signaling pathway. The detailed functional analysis of the dysregulation of adenosine signaling with special emphasis on HPV-driven tumors, however, is beyond the scope of this study and warrants further mechanistic approaches in future studies.

Remarkably, our data suggest a higher level of immune checkpoint activation in HPV-positive tumors which would therefore be expected to be associated with a worse survival compared to patients with HPV-negative tumors. This is in sharp contrast to the fact that patients with HPV-positive tumors have a significant better prognosis compared to patients with HPV-negative tumors. However, the prognosis of a malignant disease is not only determined by a single factor. HPV-positivity is associated with a HPV-specific CpG Island Methylator (CIMP) phenotype,27 indicating that a multitude of other genes apart from immune checkpoint genes are deregulated. Furthermore, HPV-negative tumors specifically and frequently harbor distinct molecular features, i.e. EGFR, TP53 (p53), FGFR1, HRAS, CCND1, and MYC genomic alterations,15,28 some of which are associated with an adverse course of the disease.29,30 Accordingly, the prognosis of a HNSCC patient cannot be traced back to a single factor but a single factor might be suited as a surrogate parameter reflecting a molecular subtype with an overall better prognosis.

We found a reduced ADORA2A methylation of CD4 T cells and monocytes in donors of older age. DNA methylation alterations as an effect of age is well described and referred to as ‘epigenetic drift’.31,32 “Immunosenescence”, aging of the immune system, is associated with chronic inflammation, defective humoral immunity, a shift from a T_{H1} to a T_{H2} cytokine profile, and increased differentiation rate of naive to memory T cells. Aging and immunosenescence are accompanied by decreased global DNA methylation and gene specific hypermethylation. Significant age-associated methylation changes have been reported for purified peripheral blood leucocytes, i.e. CD4+ T cells and CD14+ monocytes.33,34 Such age-associated methylation changes frequently affect genes involved in immune processes which are predominantly demethylated with increasing age.35-37
We are aware of two major limitations of the present study: the lack of an independent validation study and cell type-specific methylation and expression analyses. As the analysis of multiple features (beads) is generally prone to multiple testing issues, results would consequently either need to be corrected for multiple testing or validated in an independent cohort. In the present study, we aimed at reducing the risk of multiple testing artifacts by reporting the results for all beads analyzed without any selection. Further, we used median methylation and expression for dichotomization in Kaplan-Meier analyses.

Mainly, tumors represent a heterogeneous mixture of various cell types, including tumor cells, infiltrating immune cells, and tumor-associated stroma. DNA methylation patterns are well recognized tissue- and cell type-specific features associated with cell type-specific gene expression patterns. Consequently, an association between DNA methylation and gene (mRNA) expression can hardly be studied using sample material comprised of a heterogeneous mixture of cells, each showing its specific methylation and gene expression pattern. In order to address this issue, we have analyzed isolated peripheral leucocytes from healthy patients and found a significant inverse correlation between mRNA expression and methylation in CD8 and CD4 T cells in the region of a transcription start site pointing towards an epigenetic regulation of ADORA2A in these cells. However, peripheral CD8 and CD4 T cells from healthy donors are likely to be different from tumor infiltrating CD8 and CD4 T cells. Accordingly, additional studies to comprehensively analyze the association of methylation and mRNA expression levels of purified cells from tumor tissue (tumor cells, tumor-associated stroma, infiltrating CD4 T, CD8 T, Treg, and B cells as well as monocytes) are required. The correlative analyses presented in our study should therefore be interpreted with caution and neither allow for the assumption nor the ruling out of a direct epigenetic regulation in cells present in a tumor without additional experimental support. Furthermore, the correlations between methylation and mRNA expression with levels of tumor infiltration lymphocytes reported in our study are based on quantified immune cell infiltrates adopted from Li et al.\textsuperscript{16} Li and co-workers used mRNA expression signatures to assess immune cell infiltrates. The overall validity of this approach needs to be proven in additional studies. Moreover, additional studies with larger patient cohorts are needed to further investigate differences between distinct HNSCC subgroups, e.g. HPV-associated non-oropharyngeal and oropharyngeal carcinomas.

New therapeutic approaches using A2AR and CD73 inhibition have been focusing on patients with non-small cell lung cancer (NSCLC). At present, the oral antagonistic small molecule drugs CPI-444 (Corvus Pharmaceuticals, Inc. in cooperation with Genentech, Inc.) and PBF-509 (Palbofimaro SL in cooperation with Novartis), AZD4635 (HTL-1071, HTL1071, AstraZeneca) are being investigated in clinical trials including NSCLC, malignant melanoma, renal cell cancer, triple-negative breast cancer, colorectal cancer, bladder cancer, and metastatic castration resistant prostate cancer (ClinicalTrials.gov Identifiers: NCT02655822, NCT02403193, NCT03381274, NCT02740985). Efficacy of CPI-444 has been reported in 50% and 86% of patients with NSCLC and renal cell carcinoma, respectively.\textsuperscript{38} Furthermore, antagonistic monoclonal antibodies targeting CD73, including BMS-986179 (Bristol-Myers Squibb), MEDI9447 (MedImmune LCC), and IPH53 (Innate Pharma), are being developed and tested in various advanced tumors, including relapsed ovarian cancers and NSCLC (NCT02754141, NCT02503774, NCT03381274, NCT02675893).\textsuperscript{39} As reflected by the present findings, HNSCC may be a candidate for therapeutic evaluation as well and the potential of NT5E and ADORA2A methylation as predictive biomarker for response to anti-CD73 and/or anti-A2aR treatment should be tested.

### Methods

#### Analysis of mRNA expression and methylation – cell lines and isolated immune cells

Methylation data from three HPV-positive (UPCI:SCC090, 93VU-147 T, UMS:SCC047) and three HPV-negative HNSCC...
cell lines (UPCI:SCC003, UPCI:SCC036, and PCI-30) previously generated by Lechner et al. (Gene Expression Omnibus (GEO) accession number: GSE38271; National Center for Biotechnology Information, Bethesda, MD, USA) were included.\(^{40}\)

Methylation data from isolated immune cells (\(n = 97\) CD4\(^+\) T, \(n = 24\) CD8\(^+\) T, \(n = 18\) Treg, \(n = 60\) B cells, and \(n = 53\) monocytes) were obtained from three previous studies which included 26 healthy controls from Scotland (GSE87650),\(^{41}\) six

Figure 6. Association of NT5E methylation and CD73 mRNA expression with survival. Kaplan-Meier survival analyses of patients with HPV-positive (A) and –negative (B) tumors stratified according to CD73 mRNA expression and NT5E methylation determined at various sites within the gene. Median methylation and mRNA expression was used as cut-off for classifying tumors as hypo- and hyper methylated, or high and low expressing, respectively. Shown are only significant results.

Table 6. Correlation of A2aR mRNA and CD73 mRNA with T cell activation markers. Spearman’s rank between A2aR mRNA and CD73 mRNA expression with mRNA expression of genes that are commonly induced during T cell activation. Significant features are shown in boldface.
healthy Israeli women (GSE71245), and 72 healthy American volunteers (GSE59250). Matched mRNA expression data from the same samples were available from n = 23 CD4\(^+\), n = 20 CD8\(^+\), n = 4 B cells, and n = 23 monocytes. Methylation was assessed using the Infinium HumanMethylation450 BeadChip and mRNA expression levels were generated by means of HumanHT-12 V4.0 Gene Expression BeadChip (both Illumina, Inc., San Diego, CA, USA). Raw data was downloaded from the GEO webpage. Methylation values for each bead pair comprised a variant specific for the methylated and the unmethylated status and were calculated by the formula 100% x bead\(_M\) / (bead\(_M\) + bead\(_U\)).

**Patient cohort and clinical endpoints**

The results reported here are partly based on data generated by The Cancer Genome Atlas Research Network (TCGA, [http://cancergenome.nih.gov/](http://cancergenome.nih.gov/)). The TCGA cohort comprised 528 patients with histologically confirmed HNSCC collected from centers participating in the TCGA project. Due to the increasing importance of the HPV-status for the treatment of HNSCC, our study only included data on a subgroup of 279 patients adopted from Lawrence et al.\(^{15}\) For this cohort, HPV-status was determined by RNA-Seq data of the viral genes E6 and E7, thus categorizing 36 patients as HPV-positive and 243 as HPV-negative. Clinicopathological data were obtained by the TCGA Network. Clinical follow-up was assessable in all individuals (mean follow-up period 2.16 years, range 0–17.6 years). Informed consent was obtained by the TCGA Research Network from all patients in accordance with the Helsinki Declaration of 1975. Primary clinical endpoint of the study was death and time-to-death. Overall survival (OS) was defined as time between surgery and death or last contact.

**Analysis of mRNA expression and methylation – HNSCC cohort**

Data of level 2 for methylation and mRNA expression (normalized counts, n.c.) were downloaded from the TCGA webpage. A2aR and CD73 mRNA expression data were available for 20 normal adjacent (NAT) and 279 cancer samples. For the analysis of A2aR mRNA, we used RNA SeqV2 (normalized counts) data provided by the TCGA Research Network. ADORA2A and NT5E promoter methylation were available for 279 HNSCC specimens and 50 normal tissues. Methylation data were generated by means of the Infinium HumanMethylation450 BeadChip. Relative methylation levels were calculated as described above.

**Immune cell infiltrates**

Quantitative data on infiltrating lymphocytes in the HNSCC samples from the TCGA cohort (B cells, dendritic cells, CD4\(^+\) and CD8\(^+\) T cells) were adopted from Li et al.\(^{16}\) Data on FOXP3 mRNA expression from the TCGA cohort were used as a surrogate for the density of regulatory T cells.

**Statistical analysis**

Statistical analyses were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA). According to the data distribution, correlations were calculated using Pearson’s or Spearman’s correlation (Pearson’s r and Spearman’s ρ, respectively). Data on mRNA expression was logarithmized to the base of 2 for correlation analyses. Mean value comparisons were performed with the Student’s t-test (equal variances not assumed), Wilcoxon-Mann-Whitney U and Kruskal-Wallis test depending on the number of compared groups and data-distribution. Survival analyses were performed using the Kaplan-Meier method, the log rank test, and likelihood ratios. All tested factors were dichotomized according to their median in the respective groups or subgroups. OS was censored at 5 years in order to exclude deaths which were unrelated to HNSCC.\(^{27}\) P-values 0.05 were considered statistically significant.

**List of Abbreviations**

- 95%-CI: 95% confidence interval
- ADORA2A: Adenosine A2a receptor (gene symbol)
- A2aR: Adenosine A2a receptor
- CCND1: Cyclin D1 (gene symbol)
- CD73: Cluster of differentiation 73
- EGRF: Epidermal growth factor receptor (gene symbol)
- FGFRI: Fibroblast growth factor receptor 1 (gene symbol)
- HNSCC: Head and neck squamous cell carcinoma
- HPV: Human papilloma virus
- HRAS: HRas proto-oncogene, GTPase (gene symbol)
- LHR: Likelihood Ratio
- MYC: MYC proto-oncogene, bHLH transcription factor (gene symbol)
- NA: Not assessable
- NAT: Normal adjacent tissue
- n.c.: normalized counts
- NT5E: ecto-5’-nucleotidase (gene symbol)
- OS: Overall survival
- PD-1: Programmed cell death 1
- PD-L1: Programmed cell death 1 ligand 1
- TCGA: The Cancer Genome Atlas
- TP53: Tumor protein p53 (gene symbol)
- Tregs: regulatory T cells

**Disclosure Statement**

A patent application on ADORA2A methylation and other immune checkpoint genes as prognostic and predictive biomarker is pending (Inventor: Dimo Dietrich).

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