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Comparison of GeneChip, nCounter, and Real-Time PCR–Based Gene Expressions Predicting Locoregional Tumor Control after Primary and Postoperative Radiochemotherapy in Head and Neck Squamous Cell Carcinoma

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This article compares the expression and applicability of biomarkers, from single genes and gene signatures, identified in patients with locally advanced head and neck squamous cell carcinoma using the GeneChip Human Transcriptome Array 2.0, nCounter, and real-time PCR analyses. Two multicenter,

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retrospective cohorts of patients with head and neck squamous cell carcinoma from the German Cancer Consortium Radiation Oncology Group who received postoperative radiochemotherapy or primary radiochemotherapy were considered. Real-time PCR was performed for a limited number of 38 genes of the cohort who received postoperative radiochemotherapy only. Correlations between the methods were evaluated by the Spearman rank correlation coefficient. Patients were stratified based on the expression of putative cancer stem cell markers, hypoxia-associated gene signatures, and a previously developed seven-gene signature. Locoregional tumor control was compared between these patient subgroups using log-rank tests. Gene expressions obtained from nCounter analyses were moderately correlated to GeneChip analyses (median ρ = approximately 0.68). A higher correlation was obtained between nCounter analyses and real-time PCR (median ρ = 0.84). Significant associations with locoregional tumor control were observed for most of the considered biomarkers evaluated by GeneChip and nCounter analyses. In general, all applied biomarkers (single genes and gene signatures) classified approximately 70% to 85% of the patients similarly. Overall, gene signatures seem to be more robust and had a better transferability among different measurement methods. *(J Mol Diagn 2020, 22: 801—810; https://doi.org/10.1016/j.jmoldx.2020.03.005)*

Patients with locally advanced head and neck squamous cell carcinomas (HNSCC) have a heterogeneous response to radiochemotherapy (RCT), thus leading to an overall survival of only approximately 50%.[1] To date, several prognostic biomarkers have been identified[2–6] that may help to stratify patients with HNSCC regarding their response to primary RCT[7–11] or adjuvant RCT.[12–20] However, molecular methods are varying from array-based approaches to single-gene assays, thus impeding the comparability, transferability, and reproducibility of the results.

Common approaches include chip-based whole transcriptome arrays (GeneChip analyses), customized nanoString (NanoString Technologies, Seattle, WA) gene panels (nCounter analyses), and real-time PCR analyses. To date, several studies have found a high correlation between the expressions obtained by GeneChip and nCounter analyses.[21–24] An even higher correlation is stated between the gene expression measurements obtained with nCounter and real-time PCR measurements.[25,26] However, most of these studies were based on a limited number of patients or samples and were performed using versions of Affymetrix (Thermo Fisher Scientific, Santa Clara, CA) arrays considered outdated today.[27]

Therefore, the aim of this study was to compare gene expression data obtained by the three approaches, GeneChip analyses, nCounter analyses, and real-time PCR, that are available to a large number of patients and that were also based on the same RNA as starting material. We report on the two retrospective HNSCC cohorts of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG), including 327 patients in total who were diagnosed with locally advanced disease and received postoperative or primary RCT. For these cohorts, the impact of the putative cancer stem cell (CSC) markers *CD44*, *MET,* and *SLC3A2*, hypoxia-associated gene panels, and a seven-gene signature (postoperative RCT only) have previously been investigated and reported using nCounter gene expression data.[9,12,15,18] Here, the reproducibility of these results is tested using GeneChip analyses as well as real-time PCR gene expression data, which is important for conducting clinical trials, including multicenter biomarker trials, and may also play a role in meta-analyses of biomarker data.

Materials and Methods

Patients

For this study, patients of the two retrospective HNSCC cohorts of the DKTK-ROG were considered. All patients were diagnosed with locally advanced disease and were treated with either postoperative RCT[15,18,28] or primary R-CT[9,28] at one of the eight DKTK-ROG partner sites.

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The retrospective postoperative RCT cohort of the DKTK-ROG included 221 patients and was previously described.[15,18,28] Briefly, patients were treated between 2004 and 2012 with curatively intended cisplatinum-based postoperative RCT with a median dose of 64.0 Gy (range, 56.0 to 68.0 Gy) according to standard protocols. Each patient presented with a tumor stage pT4 and/or >3 positive lymph nodes and/or positive microscopic resection margins and/or extracapsular spread.

The primary RCT cohort originally consisted of 158 patients and was previously described.[9,28] Patients received primary RCT (based on cisplatinum or mitomycin-C) with a median dose of 72.0 Gy (range, 68.4 to 74.0 Gy) between 2005 and 2011.

Some of the samples of the original patient cohorts had to be omitted from the analysis because of insufficient tumor material or a too low RNA yield. The frequency of RNA assay failure was 1.2% for nCounter, 1.8% for GeneChip, and 4.6% for real-time PCR analyses. Results of both GeneChip and nCounter analyses were available for 191 patients in the postoperative RCT cohort and 136 patients in the primary RCT cohort. For 187 patients in the postoperative RCT cohort, an additional analysis with real-time PCR was performed. Both cohorts are summarized in Table 1. Expression and outcome data are available in Supplemental Table S1.

Table 1 Patient and Tumor Characteristics for the Postoperative and Primary RCT Cohorts

Characteristics	Postoperative RCT cohort	Primary RCT cohort
Follow-up, median (95% CI), months	56.7 (11.5–94.5)	54.7 (10.9–81.1)
Age, median (range), years	57.0 (24.0–75.2)	59.0 (39.2–81.9)
Dose, Gy	64.0 (56.0–68.4)	72.0 (68.4–74.0)
Sex, *n* (%)		
Male	152 (76.6)	112 (82.4)
Female	39 (30.4)	24 (17.6)
Localization		
Oropharynx	113 (59.2)	70 (51.5)
Oral cavity	53 (27.7)	23 (16.9)
Hypopharynx	25 (13.1)	43 (31.6)
UICC stage		
1		
2	7 (3.7)	
3	30 (15.7)	12 (8.8)
4	154 (80.6)	124 (91.8)
HPV16 status		
Negative	125 (65.4)	119 (87.5)
Positive	65 (34.0)	16 (11.8)
Unknown	1 (0.5)	1 (0.7)
Locoregional recurrences	28 (14.7)	54 (39.7)
Distant metastases	36 (18.9)	24 (17.7)
Deaths	61 (31.9)	70 (51.5)

Data are presented as *n* (%) of patients unless otherwise indicated. Compared with previous studies of these cohorts,[9,12,15,18] the number of included patients was reduced because of the availability of nCounter and GeneChip analyses.

HPV, human papillomavirus; RCT, radiochemotherapy; UICC, Union for International Cancer Control.

Preparation of DNA and RNA for Biomarker Analysis

Formalin-fixed, paraffin-embedded (FFPE) tissues were prepared as described previously.[9,15] After the presence of squamous cell carcinoma had been confirmed by the histologic evaluation of the hematoxylin and eosin staining, the same tissue blocks were processed under standardized procedures for biomarker investigations. In general, a tumor content of at least 5% was required for inclusion of samples in the analyses. Genomic DNA was extracted from 5-μm FFPE sections using the QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, Germany). Human papillomavirus (HPV) DNA analyses, including genotyping, were performed using the LCD-Array HPV 3.5 kit (Chipron GmbH, Berlin, Germany) according to manufacturer's instruction. Total RNA was extracted using the Tissue Preparation Kit (Siemens Healthcare Diagnostics, Erlangen, Germany), aliquoted, and stored at −80°C until required for analyses. Importantly, the RNA aliquots were originally derived from the same FFPE sections of the tumor of the respective patient, thus ensuring comparability of the starting material and subsequent gene expression analyses.

nCounter Analysis

For both cohorts, gene expression analyses were performed consecutively using nCounter technology (nanoString Technologies, Seattle, WA) as described previously.[9,15,29] Briefly, reporter and capture probes specific to the genes of interest and total RNA were pooled and incubated at 62°C for 22 hours. Then samples were kept at 4°C for a maximum of 18 hours and subsequently subjected to the nCounter system. Raw counts were logarithmized and normalized by subtracting the mean of the log-transformed counts of the reference genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11,* and *RPL37A*. For the analyses of the hypoxia-associated 15-, 26- and 30-gene signatures, the corresponding reference genes were used.[30–33] Note that the gene *DHX34* of the 30-gene signature was not available, and only the remaining 29 genes were evaluated. Because of an update of the nCounter analyses gene panel for the patients who received primary RCT, only the 158 common genes of 209 genes were considered for correlation analysis. The gene panel was composed hypotheses driven (ie, genes have been included that have previously been reported to be associated with sensitivity or resistance to RCT).[12] The considered genes are presented in detail in Supplemental Table S2,[12] and the genes of the used signatures are given in Supplemental Table S3.[12,30,32,33]

GeneChip Analysis

For whole transcriptome analyses, the Human Transcriptome 2.0 Array (Thermo Fisher Scientific) was used. For each microarray, the total RNA input amount was 10 ng. Sample processing has been performed following the instructions of the manufacturer. Quality control of the results was performed using Transcriptome Analysis Console software version 4.0 (Applied Biosystems, Waltham, MA).

Real-Time PCR

TaqMan gene expression assays were used to analyze the expression of 39 genes (Supplemental Table S2) for the postoperative RCT cohort. Of these genes, 38 were also available in the nCounter and GeneChip analyses. real-time PCR was performed as described previously[15] using the ABI StepOne Plus RT-PCR System (Life Technologies, Carlsbad, CA). All real-time PCR reactions were performed as duplicates and set up using a pipetting robot (QIAgility, Venlo, the Netherlands). Per patient, a single PCR plate was used to ensure comparability between the gene expression values. Gene expression values were logarithmized and then normalized to the mean of the reference genes *ACTR3*, *NDFIP1*, *RPL37A*, *B2M*, *GNB2L1*, *RPL11* or of the reference genes of each hypoxia gene classifier.

Clinical End Points, Data Processing, and Statistics

The correlation among gene expressions obtained by nCounter, GeneChip, and real-time PCR analyses was evaluated by the Spearman rank correlation coefficient for each gene individually. Locoregional tumor control (LRC) was measured from the first day of RCT until the event occurred or the patient was censored. Survival curves were estimated by the Kaplan-Meier method. Differences in LRC between the stratified patient groups were evaluated by log-rank tests. The impact of prognostic parameters on LRC was evaluated using univariable Cox proportional hazards regression.

The cut-offs for patient stratification that were previously defined for the CSC markers based on nCounter analyses[9,15,29] were transferred to the GeneChip data by linear regression, using the GeneChip expressions as the dependent variable and the corresponding nCounter expressions as the independent variable, to estimate their robustness. In addition, new cut-offs were defined for the GeneChip data independent of the previous studies using the same method as in previous studies[9,15] to assess whether any relevant cut-off can be identified.

On the basis of the expressions of genes in hypoxia-associated gene signatures, tumors were classified as more or less hypoxic.[30–33] These classifications were performed using k-means clustering. The classification results were compared among nCounter analyses, GeneChip analyses, and real-time PCR using cross tables.

For patients with HPV16 DNA–negative tumors treated by postoperative RCT, a seven-gene signature has previously been developed[12] based on nCounter analyses. To test the reproducibility of the signature with GeneChip analyses, gene expression data were z-normalized and a metagene was created (median expression of *SERPINE1*, *ACTN1*, *INHBA,* and *P4HA2*) for each patient. Furthermore, the signature included the binary clinical parameters extracapsular extension (0/1) and the localization oral cavity (no/yes). The prognostic index of the corresponding Cox model for LRC was used to define risk groups with a cut-off of 0.37 as described previously.[12] All presented analyses and the described statistical tests were performed using SPSS Statistics version 25 (IBM Corporation, Armonk, NY) except for the Kaplan-Meier analyses and the corresponding log-rank tests that were performed using the library lifelines in Python Language Reference version 2.7 (Python Software Foundation, Fredericksburg, VA). Two-sided tests were performed. For both patient cohorts, five biomarkers were evaluated (postoperative RCT: *SLC3A2*, *MET*, and 15- and 26-gene hypoxia signature, seven-gene signature; primary RCT: *SLC3A2*, *CD44*, and 15-, 26- and 30-gene hypoxia signature) (Table 2). Hence, accounting for multiple testing, $P < 0.01$ was considered as statistically significant.

Results

Comparison of Methods for All Available Genes

The comparison of the different gene expression measurements was independently performed for both retrospective HNSCC cohorts of the DKTK-ROG. The Spearman rank correlation coefficient between the gene expressions obtained by nCounter analyses and GeneChip analyses was evaluated for each of the 158 genes.

For the postoperative RCT cohort, the median correlation was 0.68 (95% CI, 0.11–0.89) in the postoperative R-CT cohort (Figure 1A). The correlations of the 38 genes available from real-time PCR were analyzed for the postoperative RCT cohort in detail. A higher median correlation of 0.84 (95% CI, 0.54–0.96) (Figure 1C) was observed between real-time PCR and nCounter analyses, whereas a median correlation of 0.67 (95% CI, 0.35–0.86) (Figure 1D) was found between real-time PCR and GeneChip analyses. Between nCounter and GeneChip analyses, a median correlation of 0.73 (95% CI, 0.22–0.88) was observed among these 38 genes (data not shown).

For the primary RCT cohort, both methods had a median correlation of 0.69 (95% CI, 0.07–0.90) (Figure 1B).

CSC Markers

The putative CSC markers *SLC3A2*, *MET* (only postoperative RCT cohort), and *CD44* (only primary RCT cohort) were previously evaluated using nCounter analyses.

Table 2 Correlation between nCounter and GeneChip Analysis of Genes Used for Patient Stratification and Corresponding Cox Proportional Hazards Regressions for Locoregional Control

			nCounter		GeneChip	
Parameter	$\tilde{\rho}$	$\bar{\rho}$	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Postoperative RCT						
SLC3A2	0.56	0.56	2.15 (1.23–3.76)	**0.007**	11.4 (0.74–175)	0.082
MET	0.87	0.87	2.14 (1.31–3.48)	**0.002**	3.63 (1.94–6.79)	**< 0.001**
15-Gene hypoxia signature	0.76	0.73	3.57 (1.36–9.41)	**0.010**	2.84 (1.31–6.16)	**0.008**
26-Gene hypoxia signature	0.70	0.67	9.42 (2.23–39.8)	**0.002**	3.48 (1.60–7.55)	**0.002**
7-Gene signature	0.76	0.68	5.52 (2.21–13.8)	**< 0.001**	3.93 (1.68–9.21)	**0.002**
Primary RCT						
SLC3A2	0.69	0.69	1.72 (1.16–2.53)	**0.007**	2.48 (0.54–11.4)	0.24
CD44	0.72	0.72	1.41 (0.90–2.20)	0.14	1.09 (0.88–1.36)	0.44
15-Gene hypoxia signature	0.76	0.75	1.34 (0.77–2.34)	0.30	1.39 (0.82–2.38)	0.23
26-Gene hypoxia signature	0.71	0.67	1.39 (0.77–2.52)	0.28	1.58 (0.92–2.69)	0.095
30-Gene hypoxia signature	0.69	0.66	1.48 (0.84–2.62)	0.18	0.87 (0.50–1.51)	0.62

Significant results are marked in bold. For all patients, the median ($\tilde{\rho}$) and mean ($\bar{\rho}$) Spearman correlation coefficients between GeneChip- and nCounter-based gene expressions and the HRs of the univariable Cox proportional hazards regressions with their 95% CIs are shown for the used classifiers. The upper part shows the results of the postoperative RCT cohort, and the bottom part shows the results of the primary RCT cohort.

HR, hazard ratio; RCT, radiochemotherapy.

With the use of these biomarkers, it was possible to stratify the patients of each cohort into subgroups that differed significantly regarding LRC.[9,15,29]

In univariable Cox proportional hazards regression analyses based on the gene expression data of the GeneChip analyses, *MET* remained a significant prognostic marker for LRC in the postoperative RCT cohort ($P < 0.001$) (Table 2). For the primary RCT cohort, neither *SLC3A2* nor *CD44* was significantly associated with LRC using the GeneChip analyses data set.

To stratify patients into different risk groups, two methods were applied: the available cut-offs from the nCounter data were transferred to the GeneChip data, and the cut-offs were newly defined on the GeneChip data by a method described previously[9,15,29] (Supplemental Table S4). The corresponding Kaplan-Meier estimates of LRC are presented in Supplemental Figure S1 (postoperative RCT) and Supplemental Figure S2 (primary RCT). The cut-off for *MET* could be successfully transferred from nCounter to GeneChip

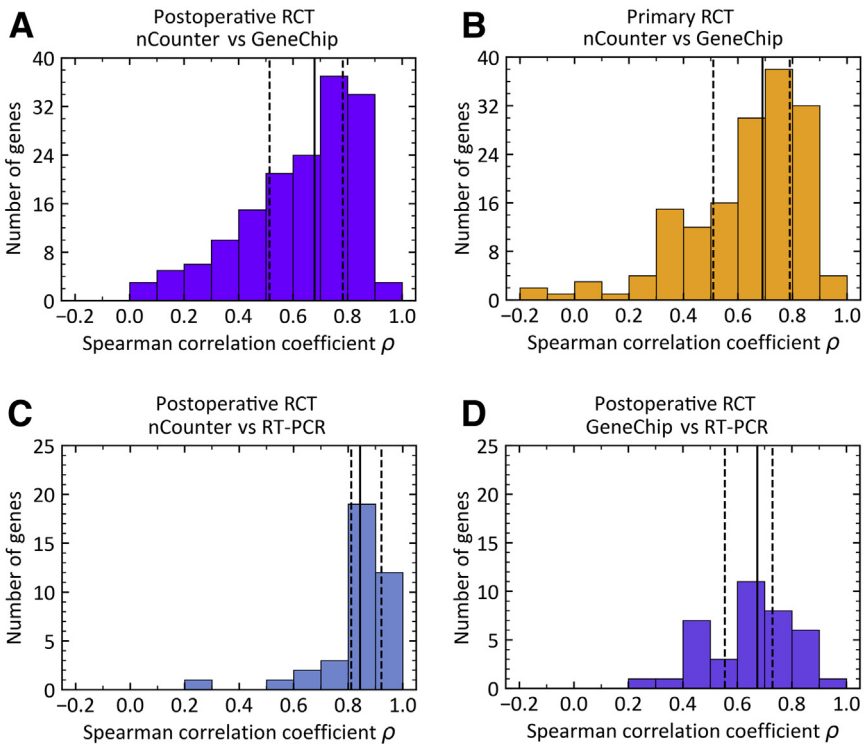

Figure 1 Correlation of gene expressions between different measurements. **A**, **C**, and **D:** Results for the postoperative radiochemotherapy (RCT) cohort. **A:** Correlation between nCounter and GeneChip analyses. **B:** Correlation between the nCounter and GeneChip for the patients treated with primary RCT. **C:** Correlation between real-time PCR and nCounter. **D:** Correlation between real-time PCR and GeneChip. **Solid lines** indicate the median correlation; **dashed lines**, the lower and upper quartiles.

data ($P < 0.01$ for both methods), whereas the cut-offs for *SLC3A2* and *CD44* could not be validated. Newly defined cut-offs for *SLC3A2* and *CD44* improved patient stratification but did not lead to statistically significant differences in LRC.

Hypoxia-Associated Gene Signatures

For the postoperative RCT cohort, the median correlations between nCounter and GeneChip analyses of the genes within the 15-gene and the 26-gene hypoxia-associated signature[30–32] were 0.76 and 0.70, respectively (Table 2). For the real-time PCR results, the median correlations with the nCounter analyses results were higher (15-gene signature: 0.89, 26-gene signature: 0.83) than with the GeneChip analyses (15-gene signature: 0.62, 26-gene signature: 0.67). For the primary RCT cohort, median correlations between nCounter and GeneChip analyses of 0.76, 0.71, and 0.69 were obtained for the 15-, 26-, and 30-gene signature,[30–33] respectively (Table 2).

Patient stratifications based on the classification of tumors as more or less hypoxic found similar results for nCounter and GeneChip (Table 2, Figure 2, and Supplemental Figures S3 and S4). Both analyses individually led to stratifications with significant differences in LRC for the postoperative RCT cohort. In general, all signatures classified approximately 70% of the patients similarly (Table 3). Classifications obtained by nCounter analyses and real-time PCR were most similar, leading to almost identical Kaplan-Meier estimates of LRC (Figure 2 and Supplemental Table S5).

Seven-Gene Signature

In addition to CSC markers and hypoxia-associated gene signatures, we recently developed a prognostic seven-gene signature for patients with HPV16 DNA–negative tumors treated by postoperative RCT.[12] The expressions of the seven included genes had a median correlation of 0.76 between nCounter and GeneChip analyses (Table 2). The

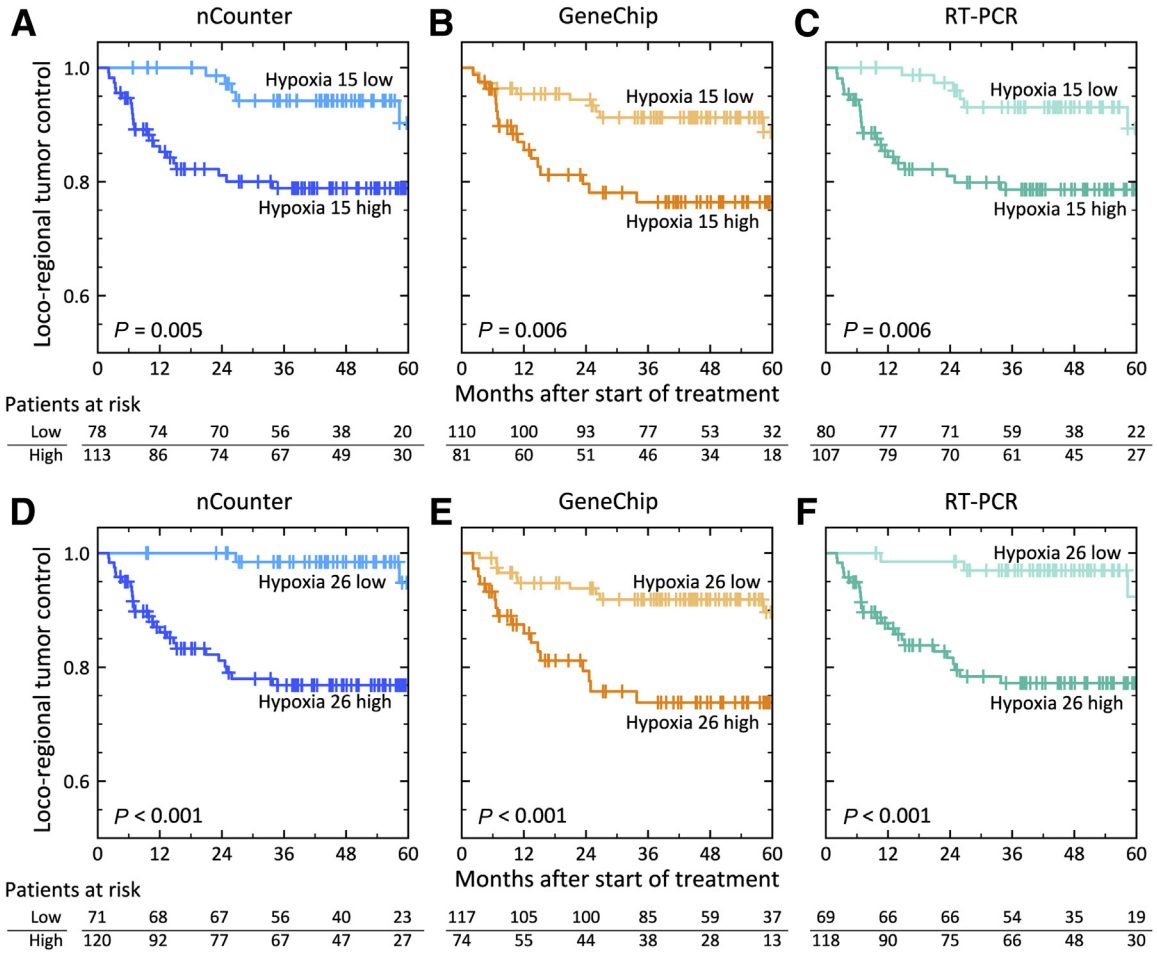

Figure 2 Kaplan-Meier estimates of locoregional tumor control for the patients treated with postoperative radiochemotherapy stratified by their hypoxia status using hypoxia-associated gene signatures. The gene expressions were obtained by nCounter (**A** and **D**), GeneChip (**B** and **E**), and real-time PCR (**C** and **F**) analyses. **A**–**C**: The stratification based on the 15-gene hypoxia-associated signature.[30,31] **D**–**F**: The stratification based on the 26-gene signature.[32] *P* values originate from log-rank tests.

Table 3 Cross Tables of Patient Stratifications Determined from nCounter and GeneChip Analysis

Stratification based on nCounter gene expression data	Postoperative RCT		Primary RCT	
	Stratification based on GeneChip gene expression data			
	Low	High	Low	High
	15-Gene Hypoxia Signature			
Low, *n*	69	9	49	5
High, *n*	41	72	34	48
	26-Gene Hypoxia Signature			
Low, *n*	63	8	41	5
High, *n*	54	66	35	55
	HPV16 DNA—Negative 7-Gene Signature		30-Gene Hypoxia Signature	
Low, *n*	59	9	46	6
High, *n*	10	47	38	46

The rows show the classification results of the nCounter-based gene expressions, whereas the columns show the classification based on the GeneChip gene expression data. The right part gives the results of the postoperative RCT cohort, and the left part gives the results of the primary RCT cohort.

HPV, human papillomavirus; RCT, radiochemotherapy.

seven-gene signature was applied to stratify the HPV16 DNA—negative patients of the postoperative RCT cohort into groups of low and high risk of locoregional recurrence, using gene expressions from nCounter (stratification 1) and GeneChip analyses (stratification 2). Eighty-five percent of the patients were classified to the same group using both analyses (Table 3), and significant differences in LRC were observed for both methods ($P < 0.001$) (Supplemental Figure S5).

Discussion

In this study, gene expressions and the prognostic value of previously studied biomarkers were compared between nCounter and GeneChip analyses for patients with locally advanced HNSCC treated by postoperative RCT[15,18,28] or primary RCT.[9,28] A moderate to high correlation between the gene expression measurement methods (median Spearman correlation of approximately 0.68) was obtained. Stratification of patients for prognosis of LRC based on the hypoxia-associated gene signatures and the seven-gene signature found similar differences in LRC for the three methods, whereas for putative CSC markers (single genes) a larger variability was observed.

In an earlier study, the results of the nanoString nCounter System were compared with gene expressions measured by the Affymetrix Human Genome U133 Plus 2.0 GeneChip for 68 paired samples of colorectal cancer. For more than 400 genes a mean correlation of 0.50 was found,[34] which is slightly lower compared with the results of our study. In another study, a gene signature associated with LRC for patients with follicular lymphoma, containing 95 genes, was developed based on the Human Genome U133 Plus 2.0 GeneChip (Affymetrix) and gene expression profiles of 134 patients.[35] The gene expressions were also measured using nCounter analyses for 53 patients. Only 23 of the gene expressions had a correlation coefficient >0.75. Again, the correlations obtained in our study were slightly higher, which may be caused by the differing Affymetrix GeneChip or the larger patient numbers.

In this study, differences in LRC between risk groups defined by gene signatures were of similar magnitude for nCounter, GeneChip, and real-time PCR analyses, whereas the results of single genes had more variability. For single genes, only the patient stratification of the putative CSC marker *MET* could be successfully transferred between nCounter and GeneChip data. Significant differences in LRC were observed for both methods. For *CD44* and *SLC3A2*, new cut-offs for the GeneChip data improved patient stratification, but still no significant differences in LRC were observed. In addition, these new cut-offs led to differing patient assignments to the risk groups compared with those obtained for nCounter data. Between 18.8% and 43.3% of the patients were assigned to different risk groups based on these cut-offs. Of note, linear regression may not always be the optimal method for transferring the cut-offs because nonlinear relationships between nCounter and GeneChip data may also be observed. On the other hand, the hypoxia-associated signatures and the seven-gene signature could be transferred between nCounter and GeneChip data, showing significant differences in LRC between the risk groups for both methods and a similar patient assignment of the postoperative RCT cohort. Although patient classification by the seven-gene signature was stable for both methods, up to 30% of the patients were assigned differently for the hypoxia-associated gene signatures. In our analysis, these patients constitute an intermediate-risk group (ie, tumors that were classified as less hypoxic by both methods had the highest LRC, tumors that were classified as more hypoxic by both methods had

the lowest LRC, and tumors that were differently classified had an intermediate LRC). However, identification of these three patient groups was not possible solely on nCounter or GeneChip data (eg, by increasing the number of clusters). This finding suggests that for the individual methods, clustering into two groups with more or less hypoxic tumors is a reasonable approach.

Our results are in line with previously reported findings[36] in which different gene classifiers were applied (eg, single genes and gene signatures using microarray data from five different studies in breast cancer). Even though the prognostic values of the single genes were similar to the gene signatures' results, the classifiers of the signatures were more stable. In a recent study, 962 different gene signatures were compared with respect to functional (same pathways) or compositional (same genes) overlap. Functional redundancy especially was found to be prominent in the signatures, ensuring that the respective pathways are covered despite tumor heterogeneity.[37] Again, this finding indicates that gene panels may be more stable and robust for patient stratification than single genes only.[23]

Real-time PCR is often referred to as clinical standard for RNA analyses.[38–40] For the postoperative RCT cohort in our study, analyses of the 15-gene and the 26-gene hypoxia signatures based on real-time PCR were available. The highest median correlations were observed between these real-time PCR expressions and nCounter analyses, whereas correlations to GeneChip analyses were lower, which is in line with the literature.[25,26] The high correlation may be further improved by the probe design of the nCounter system. Here, the probe sets were designed based on the TaqMan assays (ie, ensuring the coverage of similar gene regions). The lower correlations between GeneChip analyses and real-time PCR (Figure 1) may be explained by the differing technology. First, only one TaqMan Assay is usually being used, which is covering certain parts of the gene up to specific isoforms. In contrast, GeneChip includes several primer pairs covering different regions of the genes. If one isoform is dominating and thereby potentially masking the effects of other isoforms, this may affect the measured gene expression. In addition, one also has to consider that FFPE material has been used, which is very likely to contain fragmented (ie, short) RNA. This occurrence may lead to inconsistent results when only one set of primers or probe pairs is used compared with multiple probes[34] or GeneChip arrays, which are based on multiple primer pairs.

To investigate discrepancies between the methods in more detail, we identified those genes that for both cohorts had correlations <0.4 between GeneChip and nCounter analyses, resulting in 11 genes: *ARNT*, *BSG*, *EPOR*, *ERCC4*, *FANCA*, *MAP2K2*, *MRGGBP*, *PSMD9*, *PTEN*, *RMI2,* and *XRCC4*. From a statistical point of view, these low correlations may in part be explained by low expressions in nCounter analyses, close to the negative controls and thereby including substantial noise (*BSG*, *EPOR*, *ERCC4*), or by a low variance in the expressions of one or both methods (*ARNT*, *FGF2*, *MAPK2*, *MRGBP*, *PSMD9*, *RMI2*). This finding underlines that in particular genes with a high mean expression and a high variance should be considered as reproducible prognostic or predictive biomarkers.

The biomarkers considered in this study were subjected to independent validation on different patient cohorts previously (based on nCounter analyses).[29,41] In the validation study ($n = 152$) for the postoperative RCT cohort,[29] the putative CSC markers *CD44* and *SLC3A2* had a significant association with LRC, whereas for the hypoxia signatures a statistical trend was observed. For patients who received primary RCT, validation was performed on an independent cohort of 92 patients,[41] in which *CD44* provided additional value to the clinical parameters tumor volume and p16 status. This finding indicates that next to technical reproducibility, as analyzed in this article, independent validation on additional data sets is required. Overall, the considered biomarkers had substantially larger effect sizes on the postoperative RCT cohort than on the primary RCT cohort (Table 2). This may be due to a heterogeneous patient population in the RCT cohort comprising a large range in tumor volume and by masking the impact of hypoxia by other radiobiological parameters driving early recurrence, in particular high CSC numbers in the most advanced tumors, as discussed previously.[9] The presented results will be further validated on prospectively collected data sets [eg, on the HNprädBio trial of the DKTK-ROG (*www.clinicaltrials.gov*; NCT02059668)] in the future.

In this study, different technologies for the measurement of gene expressions were compared for patients with locally advanced HNSCC who were treated with postoperative R-CT or primary RCT gene signatures led to more robust patient stratifications than single genes and had a better transferability among the different measurement methods. Gene expressions measured by nCounter analyses and real-time PCR had a higher correlation compared with GeneChip analyses, which is likely due to their higher dynamic range[42] and fewer steps during sample preparation.[43] The results suggest that meta-analyses of biomarker data have to be conducted carefully. Although gene signatures may lead to more robust patient stratifications, single-gene assays largely depend on the method used. In case of multicentric trials, biomarker studies should be performed preferably with the same technology or in a central laboratory and should consider the future clinical practicability.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2020.03.005*.

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