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Gene signatures and expression of miRNAs associated with efficacy of panitumumab in a head and neck cancer phase II trial

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ABSTRACT

Objective: Platinum-based chemotherapy plus the anti-EGFR monoclonal antibody (mAb) cetuximab is used to treat recurrent/metastatic (RM) head-neck squamous cell carcinoma (HNSCC). Recently, we defined Cluster3 gene-expression signature as a potential predictor of favorable progression-free survival (PFS) in cetuximab-treated RM-HNSCC patients and predictor of partial metabolic FDG-PET response in an afatinib window-of-opportunity trial. Another anti-EGFR-mAb (panitumumab) was used as the treatment agent in RM-HNSCC patients in the phase II PANI01trial. PANI01 tumor samples were analyzed using functional genomics to explore response predictors to anti-EGFR therapy.

Materials and methods: Whole-gene expression and real-time PCR analyses were applied to pre-treatment samples from 25 PANI01 patients. Three gene signatures (Cluster3 score, RAS onco-signature, microenvironment score) and seven selected miRNAs were separately analyzed for association with panitumumab efficacy.

Results: Cluster3 expression levels had a profile with a significant bimodal separation of samples ($P = 3.08\,E-13$). Higher RAS activation, microenvironment score, and miRNA expression were associated with low-Cluster3 patients. The same biomarkers were separately associated with PFS. Patients with high-Cluster3 had significantly longer PFS than patients with low-Cluster3 (median PFS: 174 versus 51 days; log-rank $P = 0.0021$). ROC analysis demonstrated accuracy in predicting PFS (AUC = 0.877).

Conclusions: Despite differences in clinical settings and anti-EGFR inhibitors used for treatment, response prediction by the Cluster3 signature and selected miRNAs was essentially the same. Translation into a useful clinical assay requires validation in a broader setting.

Introduction

Inhibition of the epidermal growth factor receptor (EGFR) signaling using the monoclonal antibodies (mAbs) cetuximab and panitumumab or tyrosine kinase inhibitors including afatinib and erlotinib are established therapeutic approaches for advanced non-small cell lung and colorectal carcinomas. Intensive studies of hematological and breast cancers have indicated the potential value of whole-transcriptome analysis using high-throughput technologies to gain insights into tumor biology and creation of prognostic and/or predictive models.

HNSCC is diagnosed in approximately two-thirds of patients at advanced stages (stage III/IV). Relapse within two years after primary

Abbreviations: EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; RM, recurrent/metastatic; HNSCC, head-neck squamous cell carcinoma; PFS, progression free survival; ADCC, antibody-dependent cellular cytotoxicity; FFPE, formalin-fixed paraffin-embedded; GEO, Gene Expression Omnibus; ROC, Receiver Operating Characteristic; AUC, area under a ROC curve; ECOG, Eastern Cooperative Oncology Group; WHO, World Health Organization; HR, hazard ratio; CI, confidence interval

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treatment occurs in 27–50% of these patients. Systemic treatment in these recurrent/metastatic (RM) patients is typically platinum-based chemotherapy plus cetuximab [1], which is the evidence-based first-line treatment of patients with RM-HNSCC [2]. The response rate is approximately 40% with a small portion of these patients having a durable response.

An increasing number of gene expression (GE) datasets for head and neck squamous cell carcinoma (HNSCC) [3] are enabling the identification of promising molecular signatures. However, few clinically applicable predictors of the response of HNSCC patients are available, reflecting the relatively limited number of available datasets. We previously performed a meta-analysis of 20 datasets seeking to clarify the molecular sub-classification of HNSCC. Of the six GE subtypes, Cluster3 was associated with poor outcome [4], however exploration of drug sensitivity using data from the cell lines in the cancer genomic project [5] revealed that the high expression of Cluster3 predicted afatinib (a small molecule that irreversibly inhibits the tyrosine kinase activity of epidermal growth factor receptor [EGFR] and human epidermal growth factor receptor 2 [HER2]) sensitivity.

Then, we analyzed the GE profiles of a retrospective series of HNSCC patients treated with platinum-based chemotherapy plus cetuximab [6]: (i) we observed that patients displaying prolonged progression free survival (PFS) consistently belong to Cluster3 [4] and Basal [7] subtypes, while short PFS is characterized by an over-activation of RAS signaling; (ii) we computationally predicted a significant association between the high expression of Cluster3 and sensitivity to afatinib in patients with prolonged PFS [6]. Furthermore, in a recent window of opportunity trial, we experimentally confirmed the association between the high expression of Cluster3 and the partial response to afatinib [8]. Finally, focusing on the integration of microRNA (miRNA) and GE for the prediction of cetuximab response, we identified a potential predictive role of selected miRNAs [9].

Panitumumab is a fully human anti-EGFR-mAb. It was investigated as a single agent in the phase II PANI01 trial) in platinum-resistant RM-HNSCC patients [10]. The primary endpoint of response rate was not reached, although impressive PFS (median 2.6 months; 95% CI: 1.7–3.7) and estimated overall survival (median 9.7 months; 95% CI: 6.3–17.2) were observed. Durable responses were limited.

Here we evaluated the previously identified potential predictors of the response to EGFR inhibitors on the available tumor samples from the PANI01 trial to further gain insights into their predictive role for PFS.

Patients and methods

Phase II PANI01 trial

Patients enrolled in the PANI01 trial were pre-treated with platinum-based chemotherapy without cetuximab. Previous cetuximab treatment within a curative chemo-radiotherapy schedule or as first-line treatment was only allowed in the absence of disease progression for at least 3 months. Panitumumab was administered intravenously at a dose of 6 mg/kg on days 1 and 15 of a 28-day cycle. The primary and secondary endpoints were response rate and PFS, respectively. The study was approved by the Independent Ethics Committee of the Oncology Institute of Southern Switzerland, Bellinzona (Approval Number 2370). Further details are provided elsewhere [10].

Gene-expression profiling

Formalin-fixed, paraffin-embedded (FFPE) tumor specimens were used, including 20 samples from primary tumors and five from RM. RNA extraction, quality control, and hybridization on DASL microarrays (Illumina, San Diego, CA, USA) were performed essentially as previously described [6]. Microarray data were deposited and are available at the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE102995).

Bioinformatics analyses

A score was determined according to the Pearson correlation between the gene expression profile of each sample and the centroids for Cluster3 and Basal subtypes of De Cecco [4] and Keck [7] classification, respectively. Scatter plot analysis depicting the relationship between Cluster3 and Basal centroids was performed by R graphics package (function: *plot*). In order to transpose these variables into a clinical decision, patients should by stratified into defined groups and, to this purpose, it is necessary to identify a cutoff point. The threshold to dichotomize high- and low-Cluster3 values was defined using the Cutoff Finder tool [11] a web application freely available at http://molpath.charite.de/cutoff and implemented as Java Server Pages (JSPs) that connect the R packages and the TCP/IP server Rserve [12]. Cutoff determination was performed by fitting a mixture model to the distribution of the biomarker, a method optimized for molecular traits showing bimodal shaped distribution. A two-Gaussian model built through the R package flexmix (function: *flexmix*) [13] is fitted to the histogram distribution. The cutoff value corresponds at the point where the probability density functions of the mixing distribution coincide.

RAS oncosignature analysis was performed as described in [9] using single-sample gene set enrichment analysis (ssGSEA) projection [14] through GenePattern [15] available at http://software.broadinstitute.org/cancer/software/genepattern# to assess gene set activation scores in each sample of our case material. According to the instructions described in ssGSEAProjection documentation, GCT files containing the gene expression data were created as input files and RAS oncogenic signature present in c6 collection of Molecular Signatures Database (MSigDB) was used for ssGSEA.

In order to portray the tumor microenvironment composed of numerous distinct non-cancerous cell types, we assessed the microenvironment score using xCell, a novel method based on tissue transcriptome profiles that integrates the advantages of gene set enrichment with deconvolution approaches [16] available at http://xcell.ucsf.edu/. The method relays on about 500 gene signatures corresponding to 64 cell types spanning multiple immunity cells, hematopoietic progenitors, epithelial cells, and extracellular matrix cells and the composite microenvironment score was defined as the sum of all immune and stroma cell types.

miRNA Real-time PCR

RT-qPCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark). The miRNA analyzed (in brackets respective ID of each single Exiqon assay) were: hsa-miR-199a-3p (MIMAT0000232, ID: YP00204536), hsa-miR-199b-5p (MIMAT0000263, ID: YP00204152), hsa-miR-199a-5p (MIMAT0000231, ID: YP00204494), hsa-miR-130b-3p (MIMAT0000691, ID: YP00204317), hsa-miR-140-5p (MIMAT0000431, ID: YP00204540), hsa-miR-214-3p (MIMAT0000271, ID: YP00204510), and hsa-miR-34a-5p (MIMAT0000255, ID: YP00204486); one miRNA that resulted not modulated in the microarray analysis [9], hsa-miR-451a (MIMAT0001631, ID: YP02119305), was used as control. Total RNA (20 ng) were reverse-transcribed at 42 °C for 60 min, followed by heat inactivation at 85 °C for 5 min using a poly-T primer containing a 5′ universal tag. The resulting cDNA was diluted 1:80 in nuclease-free water and 8 µl were amplified in 20 ml at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 60 s. Normalization was performed with SNORD44 (ID: YP00203902). miRNA expression levels were quantified using the QuantStudio 12K flex sequence detection system (Life Technologies, Carlsbad, CA, USA), and the threshold cycle (Ct) for each sample was determined. ABI SDS 2.4 software (Life Technologies) was used to recover the data, and the relative expression was calculated using the comparative $-\Delta Ct$ method.