IJC
International Journal of Cancer

A comprehensively characterized large panel of head and neck cancer patient-derived xenografts identifies the mTOR inhibitor everolimus as potential new treatment option

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Patient-derived xenograft (PDX) models have shown to reflect original patient tumors better than any other preclinical model. We embarked in a study establishing a large panel of head and neck squamous cell carcinomas PDX for biomarker analysis and evaluation of established and novel compounds. Out of 115 transplanted specimens 52 models were established of which 29 were characterized for response to docetaxel, cetuximab, methotrexate, carboplatin, 5-fluorouracil and everolimus. Further, tumors were subjected to sequencing analysis and gene expression profiling of selected mTOR pathway members. Most frequent response was observed for docetaxel and cetuximab. Responses to carboplatin, 5-fluorouracil and methotrexate were moderate. Everolimus revealed activity in the majority of PDX. Mutational profiling and gene expression analysis did not reveal a predictive biomarker for everolimus even though by trend *RPS6KB1* mRNA expression was associated with response. In conclusion we demonstrate a comprehensively characterized panel of head and neck cancer PDX models, which represent a valuable and renewable tissue resource for evaluation of novel compounds and associated biomarkers.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer world wide.[1] Despite improved survival, especially through the introduction of targeted agents it remains a devastating disease. A potential patient stratification by means of predictive biomarkers has not been successfully established for clinical routine use.[2] The majority of anticancer drugs tested in early clinical trials failed to show a clinical benefit. Although preclinical drug evaluation in cell lines has been a useful tool for mechanistic exploration, those cell lines have repeatedly failed to predict clinical impact[3] and cell lines of HNSCC, especially HPV positive lines have proven difficult to establish. Patient-derived xenografts (PDX) have been recognized to better predict clinical outcome, since this preclinical model shares similar histology, comparable gene expression patterns over several passages and retain tumor heterogeneity as seen in the primary specimen.[4–6] We aimed to establish an extensive number of patient-derived xenograft models of HNSCC for translational research, preclinical drug screening and biomarker identification and validation. In a first step we characterized the established models for compounds used in standard of care (SoC) treatment to identify resistant tumors needing alternative treatment options and reanalyze response to SoC for predictive signatures in the available gene expression and mutational patterns. EGFR inhibition has become an important part of HNSCC treatment schedules, however still with limited success. Aim of our translational studies was the search for a rational alternative targeted treatment. Everolimus is one of two mTORC1 inhibitors, which have been successfully introduced into clinical routine for the treatment of renal cell carcinoma, breast cancer and neuroendocrine tumors. However, not all patients experience a benefit from mTOR inhibition and biomarker identification for patient selection has been defined as a crucial issue.[7] We aimed to evaluate the established PDX models for treatment response to everolimus and correlate our findings with tumor biology. By this way

Key words: xenograft, head and neck cancer, PDX, everolimus, PI3K, patient avatar, mTor, HNSCC
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Wilhelm-Sander Stiftung
DOI: 10.1002/ijc.29344
History: Received 8 June 2014; Accepted 31 Oct 2014; Online 18 Nov 2014
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What's new?
Preclinical drug evaluation in head and neck squamous cell carcinoma (HNSCC) is challenged by the inability of established cell lines to predict clinical impact. It may be possible to overcome that problem with patient-derived xenografts (PDX), which more closely reflect tumor characteristics. Here, a large collection of PDXs were established for HNSCC and tested for therapeutic response. The mTOR inhibitor everolimus was found to be active in a majority of the models. Biomarkers capable of predicting tumor response to everolimus were not identified, though increased expression of *RPS6KB1*, a member of the mTOR pathway, was common among responders.

we intended to develop a well-founded hypothesis for clinical application of this compound.

Material and Methods

Establishment of patient-derived xenografts

Patients with head and neck tumors planned for surgical treatment were approached for sample donation. Patients included in the study stated written informed consent and the study was approved by the local Institutional Review Board of Charité University Medicine, Germany (EA4/019/12). Tumor samples, which were not needed for pathological review were used for xenotransplantation. Tumor pieces of 3–4 mm were placed in RPMI media and transferred at room temperature to the animal facility. Transplantation was done on NOD.Cg-Prkdcscid *Il2rgtm1Wjl/SzJ (NSG)* mice subcutaneously within 24 hr after tumor surgery, since these mice have been advocated for the highest engraftment rate compared with other strains.[8] Additional tissue samples were immediately snap-frozen and stored at −80°C for genomic and protein analyses. All animal experiments were done in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals and of the German Animal Protection Law and approved by the local responsible authorities.[9] Samples were anonymized and given an internal number. In case of transplantation of primary tumor and metastasis from the same patient, this was indicated by A and B, respectively.

Engrafted tumors at a size of about 1cm^3 were surgically excised and smaller fragments retransplanted to naïve NMRI nu/nu mice for further passage. Next to economical consideration this strain was chosen since engrafted tumors will continue growth on less immunocompromised mouse strains and to ensure comparability of results since maximum tolerated doses was previously assessed in nude mice within our group. Within passage 1 to 3 numerous samples were conserved in DMSO for further experiments. Tumors were passaged not more than six times.

Chemosensitivity testing

Response to compounds used in clinical routine was evaluated in early passages after confirmation of histological tumor identity. For determination of chemotherapeutic response fragments of similar size were transplanted subcutaneously to a large cohort of mice. At palpable tumor size (50–100 mm^3), mice were randomized to a treatment or control group consisting of six animals each. Doses and schedules were chosen according to previous experience in animal experiments and represent the maximum tolerated or efficient doses. Applied schedules are shown in Table 1. The injection volume was 0.2 ml/20 g body weight. Treatment was continued over a period of 3 weeks unless tumor size exceeded 2 cm^3 or animals showed loss of 10% body weight. No group lost more than one animal due to toxicities during the treatment. At the end of the treatment period animals were sacrificed and tumor samples were stored in liquid nitrogen immediately.

Tumor evaluation

Animals were observed twice daily for health condition. Twice weekly, animals were evaluated for tumor size and body weight. Tumor measurement was done two-dimensional with a sliding caliper. Individual tumor volumes (V) were calculated by the formula: $V = ([\text{width}]^2 \times \text{length})/2$. Mean tumor volumes of treated in relation to mean tumor volume of control animals (T/C) were used for the sensitivity evaluation of each treatment modality after 3 weeks of treatment.

H&E staining of primary tumor and xenografts

For confirmation of tumor histology tumor tissue was embedded in Tissue-tek and 5 µm cryo sections were prepared. Samples were stained according to a standard protocol for hematoxilin eosin to ensure xenograft comparability to the original specimen. Cases with changed histological pattern were sent for pathological review and CD 20 staining was performed in order to exclude the outgrowth of lymphoproliferative disorders.

Determination of HPV status

P16 staining as surrogate marker for HPV infection was used for screening at the pathology department of Charité University Hospital on patient tumor material. To confirm and asses stable expression of HPV DNA in tumor xenografts PCR analysis for E6 and E7 was performed in p16 positive cases. Analysis was restricted to HPV-16 since this type comprises about 90% of HPV associated tumors in the oropharynx.[10] Primers and probes used were adapted from Zhao *et al.*[11] Total genomic DNA was isolated from tumor samples using DNeasy blood and tissue kit from Qiagen according to the

manufacturer's instructions. Quality and quantity assessment was accomplished using Nanodrop Spectrophotometer 1000. All samples were run in duplicate.

RNA preparation and quantitative PCR

RNA isolation was done using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Quality and quantity assessment was accomplished using Nanodrop Spectrophotometer 1000 (PeqLab). RNA was reverse transcribed using SuperScript III Reverse Transcription Kit (Invitrogen). Human gene primer/probe pairs for *MTOR* (Gene ID 2475, HS00234508), *RPS6KB1* (Gene ID 6198 HS00177357), *Akt1* (Gene ID 207, HS00178289), *FKBP1B* (Gene ID2281, HS00997682) *TSC1* (Gene ID 7248, HS1060648) and *GAPDH* (Gene ID 2597 HS99999905) and TaqMan Fast Master Mix obtained from Applied Biosystems were used according to the manufacturer's instructions and amplifications were carried out on the Applied Biosystems 7500 Real-time PCR cycler. GAPDH was used as housekeeping gen.

All samples we run in duplicate. Results are displayed as delta CT values as relative quantification.

Table 1. Compounds, dosage, application schedule and route of application of evaluated substances in patient derived xenografts

Compound	Dose	Schedule	Application route
Docetaxel	12.5 mg kg^{-1}	Once weekly x3	iv
Carboplatin	75 mg kg^{-1}	Once weekly x3	ip
Cetuximab	50 mg kg^{-1}	Once weekly x3	iv
5-fluorouracil	100 mg kg^{-1}	Once weekly x3	ip
Methotrexate	10 mg kg^{-1}	q3d	ip
Everolimus	4 mg kg^{-1}	d1–5 x3 weeks	po

Abbreviations: iv: intravenous, ip: intraperitoneal, po: per os, q3d: every 3rd day.

DNA Sequencing

Mutational analysis of primary tumor samples and selected corresponding xenografts was accomplished on Illuminas TruSeq Amplicon—Cancer Panel. With this panel 48 genes are targeted with 212 amplicons in a multiplexed reaction.

Table 2. Clinical characteristics of patients whose tumors led to successful establishment of patient derived xenografts

Tumor ID	TNM	UICC stage	Grading	Age	Site of tumor origin	Gender	Primary/recurrent
9619	T2N0M0	II	NA	NA	Oropharynx	Female	Recurrent
9876	T3N2cM0	IVA	G3	62	Hypopharynx	Male	Recurrent
9897	T2N2bM0	IVA	G3	58	Hypopharynx	Male	Recurrent
10110	T2N2cM0	IVA	G2	69	Tongue	Male	Primary
10114	T3N0M0	III	G3	52	Floor of mouth	Male	Primary
10159	T1N0M0	I	G2	57	Floor of mouth	Male	Primary
10309	T4N2cM0	IVA	G3	55	Oropharynx	Male	Primary
10321	T2N0M0	II	G2	65	Tongue	Male	Primary
10379	T3N2bM0	IVA	G2	39	Soft palate	Male	Primary
10511	T2N0M0	II	G2	54	Oropharynx	Male	Primary
10621	T2N2bM0	IVA	G3	61	Oropharynx	Male	Primary
10632	T2N1M0	III	G3	60	Tongue	Male	Primary
10847	T2N1M0	III	G2	71	Soft palate	Female	Recurrent
10913	T4N2bM0	IVA	G2	50	Floor of mouth	Male	Primary
10924	T3N2cM0	IVA	G2	65	Hypopharynx	Male	Primary
10927	T2N2bM0	IVA	G2	67	Oropharynx	Male	Primary
10960	T2N0M0	II	G2	63	Tongue	Male	Primary
10980	T4bN2bM0	IVB	G2	59	Soft palate	Female	Primary
11097	T4aN2bM0	IVA	G2	75	Floor of mouth	Female	Primary
11142	T2N2cM0	IVA	G3	46	Floor of mouth	Male	Primary
11143	T2N2bM0	IVA	NA	82	Oropharynx	Male	Primary
11218	T4N0M0	IVA	G2	68	Soft palate	Female	Primary
11269	T4aN2cM0	IVA	G2	71	Floor of mouth	Male	Primary
11437	T4bN2cM0	IVB	G2	56	Floor of mouth	Male	Primary
11452	T2N0M0	II	G2	75	Floor of mouth	Male	Primary
11482	T2N2bM0	IVA	G2	61	Floor of mouth	Male	Primary

All necessary reagents were purchased from Illumina. Total genomic DNA was isolated from tumor samples using DNeasy blood and tissue kit from Qiagen according to the manufacturer's instructions. About 250 ng of high quality genomic DNA (A260/280 1.8-2) were used for hybridization to a custom pool of oligos on a hybridization plate. Unbound oligos were removed using a filter capable of size selection by repeated washing followed by extension-ligation of bound oligos, which resulted in the formation of products containing the targeted regions of interest. The products were amplified using primers that add index sequences for sample mulitplexing as well as common adapters required for cluster generation. This was followed by library normalization. For cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer and heat denatured prior to MiSeq sequencing. MiSeq sequencing was carried out on Illumina MiSeq. Illumina Variant Studio 2.1 was used for sample analysis. For correlation analysis, known SNPs were excluded and only somatic mutations, which occurred with an allelic frequency >5% were considered. We have to acknowledge employing chip technology in sequencing analysis bears the risk of missing genetic variants that might occur to a minor extend in other regions of the genome as covered by the amplicons prespecified.

Primary tumors and thereof derived xenografts were evaluated in nine matched samples. For the all others, tumor DNA was isolated from the tumors of the control group animals in the chemosensitivity evaluation.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5. Response evaluation after 3 weeks of treatment using *T*/*C* values was done by two way ANOVA testing. A p value of <0.05 was considered as statistical significant. Correlation analysis was performed as Spearman rank-order correlation with a two tailed p value.

Results

In total 115 tumor samples from 89 patients with primarily diagnosed or recurrent head and neck squamous cell carcinomas were transplanted to immunodeficient mice. About 52 (45%) led to stable growth with confirmed histological appearance, whereas 63 (55%) did not grow after transplantation or resulted in outgrowth of CD20 positive lymphoproliferative disease ($n = 10$) not resembling the primary tumor. Median time to first passage was 69 days after tumor inoculation, ranging from 30 to 222 days.

From all cases, 14 tumors were classified as HPV positive by strong p16 expression in the majority of cancer cells. However, we successfully established only two PDX models (14% engraftment rate) from these HPV positive tumors. Interestingly we observed a high rate of lymphoproliferative disease in six HPV positive tumors.

PCR for viral genome of HPV type 16 showed stable expression of E6 and E7 over several xenograft generations for the two stable growing models. Twenty-nine models of HNSCC PDX were used for preclinical drug sensitivity screening after reaching 3rd passage. Patient characteristics of established PDX are summarized in Table 2. The number of models represents a clinical phase II setting considering a probability of response of 0.2 according to Simon *et al.*[12] and thereby provide representative data about single agent activity. For various tumors tissue from corresponding local lymph node metastases were transplanted. To date this resulted in three models for which we successfully established paired PDX of primary tumor as well as metastatic disease.

Validation studies

Several analyses were performed to verify, that the tumor growing in the PDX resembled the tumor characteristics found in the corresponding patient. Hematoxillin & Eosin (H&E) staining revealed high similarity of PDX and primary patient tumor, however, we observed 10 cases of changed histological pattern from squamous cell carcinoma to lymphoblastic disease in the entire cohorte. Those cases were confirmed by human specific CD20 staining as lymphoblastic cells and excluded from further analysis.

We performed NGS using the Illumina Cancer panel to identify mutational spectrum of the new models. By sequencing both, the patient tumors and the engrafted PDX we were able to follow up on how mutational patterns are consistent over several passages in patient-derived xenografts models of head and neck cancer. Figure 1 shows mutations in the majority of samples.

Mutational analysis of our cohort on the Illumina Cancer Panel revealed a mutational pattern comparable to the recently described panel of Stransky *et al.* and the large cohort evaluated within the TCGA.[13,14] We detected TP53 mutation in 20 of the 29 (69%) models and PIK3CA mutation in seven models (24%) of the cohort. Other mutations occurred with low frequency. The majority of TP53 mutations were classified as deleterious, according to the functional evaluation by Kato *et al.*,[15] which is shown in the Supporting Information.

Chemosensitivity studies

Response to treatment was very heterogenous. *T*/*C* value below 50% and significant growth inhibition to control tumors were considered as responder. Overall best response rate was observed for treatment with docetaxel with 26 of 29 (89%) responders (mean *T*/*C* value of 23) and by cetuximab with 23/29 (79%) responders (mean *T*/*C* value of 32). Even though the dosage of classical chemotherapies such as 5-fluorouracil, carboplatin and methotrexate was according to maximum tolerated dose, in general this did not result in significant growth inhibition when given as single agent as in our approach. Responders for 5-FU were 14/29 (48%), for carboplatin 13/29 (44%) and methotrexate 6/29 (20%) with mean *T*/*C* values of 59, 62 and 82 respectively. Representative study results are shown in Figure 2.

Additionally to standard of care compounds we evaluated the mTOR inhibitor everolimus. Response rate was 20/29

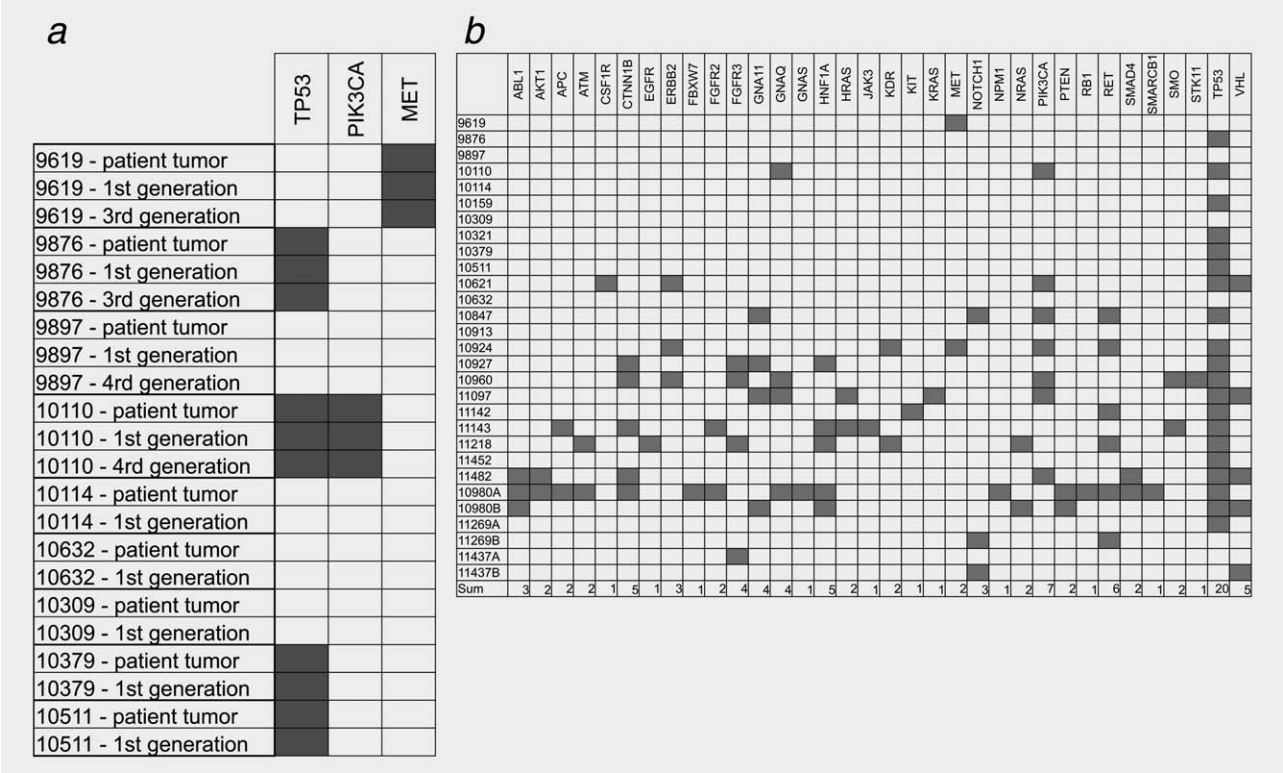


Figure 1. (*a*) showing mutational profile consistency over multiple generations for nine different patient tumors and thereof derived xenograft models. Model 10309 without any detectable mutation was identified as HPV positive. (*b*) showing the mutational profile of the entire characterized PDX panel as detected on Illumina Cancer Panel.

(68%) with a mean *T*/*C* value of 50. *T*/*C* values are shown in Figure 3.

Functional mTOR pathway analysis and correlation of response to mutational patterns

To correlate basal expression of the key members of the mTOR pathway, we analyzed quantitatively the gene expression with RT PCR. Figure 4 shows the delta CT Expression values of Akt1, mTOR, and RPS6KB1. (TSC1 and FKBP1B expression levels can be found in the Supporting Information). RPS6KB1 gene expression showed a trend to positive correlation with treatment response (T/C) values ($p = 0.0784$). Expression level of mTOR was significantly associated to expression of AKT1 ($p = 0.003$), TSC1 ($p = 0.0012$) and RPS6KB1 ($p = 0.0064$) but not to FKPB1B ($p = 0.7958$).

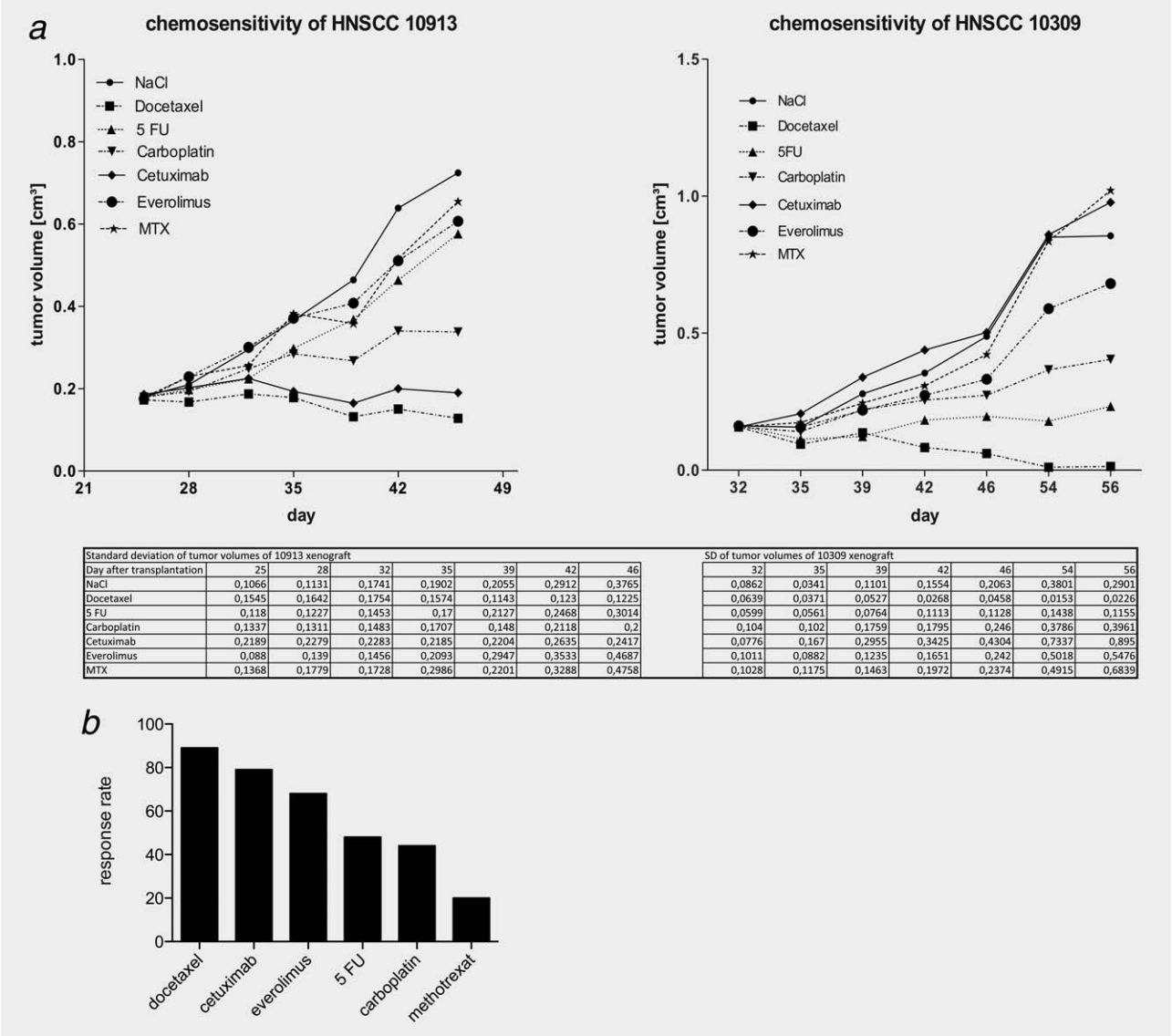
We observed the highest expression of mTOR pathway genes within the models 10110, 10980B and 11097. However, high gene expression levels of mTOR pathway members did not clearly translate into a better response to everolimus compared to models with low expression of RPS6KB1, Akt1 and mTOR such as 11142, 11482 and 11437A.

The detection of TP53 mutation did not influence treatment response to everolimus, independent whether mutations were classified as deleterious or tolerable. PIK3CA mutation has been reported to occur in up to 20% of head and neck carcinomas and activating mutations have been associated to increased pathway signaling, tumor formation and sensitivity toward PI3KCA inhibitors.[16,17] However, there was no statistical significant correlation in gene expression within the evaluated mTOR pathway and the occurrence of PI3KCA mutation as well as response to Everolimus in our models.

Discussion

Within 2 years we successfully established 52 patient-derived xenografts of head and neck squamous cell carcinoma, which to our knowledge represents the largest collection of this tumor entity. Employing PDX for biomarker studies and evaluation of new treatment modalities has been advocated as a superior preclinical model in comparison to cell lines because those models reflect the original patient tumor closer than any other preclinical model.[18–20] Furthermore, the collection of different patient tumors on xenografts reflects the diversity of HNSCC.

As others before, we were able to show that histological patterns are resembled in the xenograft tumor.[17,21,22] Furthermore we were able to show that mutational patterns are conserved over several passages within our validation studies. Even though a growing body of evidence shows similarity between original patient tumor and thereof derived xenograft tumors thorough validation for each patient-derived xenograft remains an essential issue, since patient tumor



Standard deviation of tumor volumes of 10913 xenograft							
Day after transplantation	25	28	32	35	39	42	46
NaCl	0,1066	0,1131	0,1741	0,1902	0,2055	0,2912	0,3765
Docetaxel	0,1545	0,1642	0,1754	0,1574	0,1143	0,123	0,1225
5 FU	0,118	0,1227	0,1453	0,17	0,2127	0,2468	0,3014
Carboplatin	0,1337	0,1311	0,1483	0,1707	0,148	0,2118	0,2
Cetuximab	0,2189	0,2279	0,2283	0,2185	0,2204	0,2635	0,2417
Everolimus	0,088	0,139	0,1456	0,2093	0,2947	0,3533	0,4687
MTX	0,1368	0,1779	0,1728	0,2986	0,2201	0,3288	0,4758

SD of tumor volumes of 10309 xenograft							
32	35	39	42	46	54	56
0,0862	0,0341	0,1101	0,1554	0,2063	0,3801	0,2901
0,0639	0,0371	0,0527	0,0268	0,0458	0,0153	0,0226
0,0599	0,0561	0,0764	0,1113	0,1128	0,1438	0,1155
0,104	0,102	0,1759	0,1795	0,246	0,3786	0,3961
0,0776	0,167	0,2955	0,3425	0,4304	0,7337	0,895
0,1011	0,0882	0,1235	0,1651	0,242	0,5018	0,5476
0,1028	0,1175	0,1463	0,1972	0,2374	0,4915	0,6839

Figure 2. (*a*) representative growth curves of two head and neck cancer patient-derived xenograft tumors. Treatment duration lasted for 3 weeks. One treatment group consisted of six animals. The right graph shows a HPV positive model. Underneath standard deviation of tumor volumes are given at time points of measurement. (*b*) response rate in percent for the evaluated compounds of 29 xenografts.

fragments which always include lymphocytes of the donor patient transplanted to NSG mice may result in outgrowth of transformed B-cells to form a type of lymphoproliferative disease. Within our cohort of 115 transplanted tumor samples we observed lymphoproliferative disease in 10% of transplanted samples of squamous cell carcinomas. Mouse lymphoma was ruled out by usage of human specific CD20 antibody. This phenomenon is in accordance to a report by Chen *et al.* who observed lymphoproliferative disease in 11 of 21 transplanted samples of hepatocellular carcinoma.[23] Before the introduction of NSG mice the phenomenon of evolution of lymphoproliferative disorder has not been an issue.[24] However employing NSG mice has led to superior engraftment rates and therefore remains a valuable platform with the need of thorough validation.[8]

Our collection of PDX lacks a relevant number of HPV positive tumors although a significant number of HPV positive tumors were initially transplanted. Even though it has been reported that HPV positive tumors show similar engraftment rates to HPV negative tumors, we were not able to reproduce this observation.[21] Because HPV associated tumors occur most often in tonsilar squamous cell epithelium, a per se lymphocyte rich tissue, xenotransplants from those tumors frequently gave rise to lymphoproliferative disease. Another reason for the low number of HPV positive models may arise from the study population, which were mainly elderly smokers with tumors in the oral cavity. However, for the p16 positive tumors it remains an unsolved issue why engraftment is low and it might be a similar problem as seen in the attempts of establishing HPV positive cell lines.

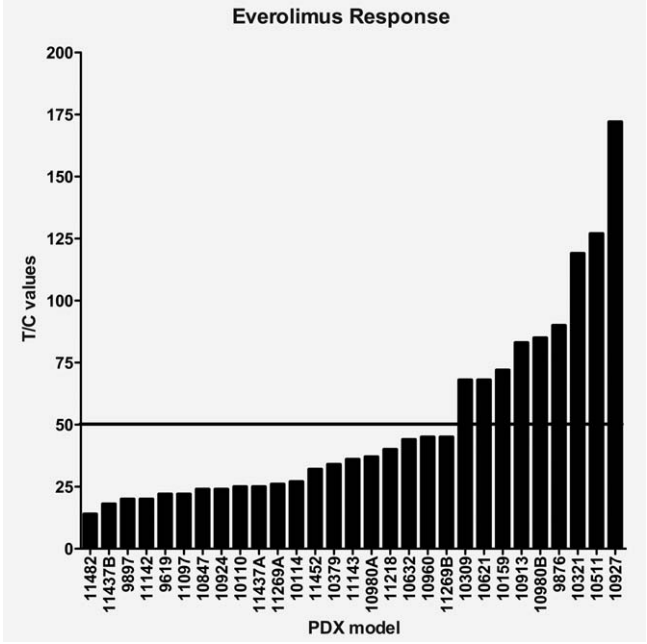


Figure 3. Everolimus response of individual patient-derived xenograft tumors expressed in *T*/*C* values. The line at *T*/*C* 50 marks the cut off for treatment responders and non responder. *T*/*C* values below 50 were considered as responders. Models indicated with A represent primary tumors and B the established model of the corresponding loco regional lymphatic metastasis.

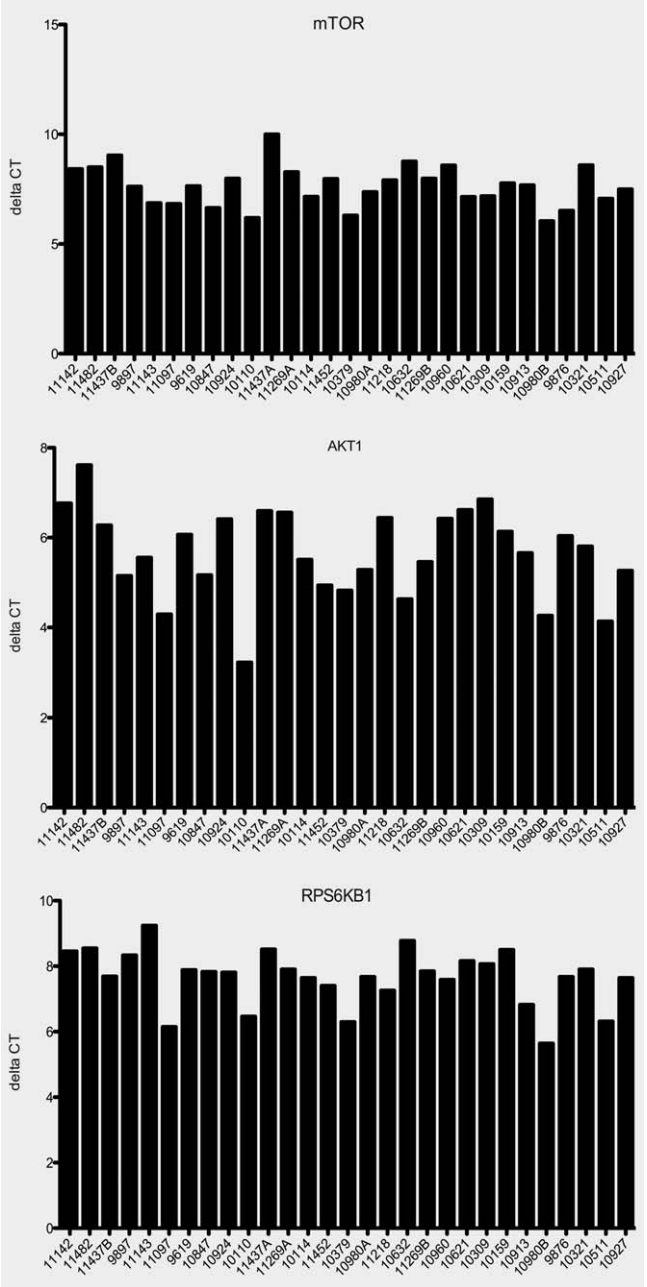


Figure 4. Delta CT values for mTOR pathway members RPS6KB1, AKT1 and mTOR sorted to Everolimus response with best responders on the left to resistant tumors on the right.

The two models, which we successfully established were positive for HPV16 and PDX tumors retained the HPV genome over several passages.

In our drug screening studies we evaluated the impact of traditionally used chemotherapeutic agents in head and neck cancer as a basis for further correlations, biomarker studies and definition of potential therapeutic partners. Highest response rate was observed for docetaxel (89%) and cetuximab (79%) and moderate activity for 5 FU (48%), methotrexate (20%) and carboplatin (44%). Prolonged treatment periods and combination treatments were beyond the scope of this study. The majority of the study population consisted of previously untreated patients, which might explain the high response rates of docetaxel and cetuximab. Appropriate *T*/*C* value cut off points for a clinical meaningful response is a controversial issue. Voskoglou–Nomikos proposed if more than one third of the evaluated animals show a meaningful response we might expect some activity in a phase II trial.[25] We choose to set the cut off point at *T*/*C* 50%. Johnson *et al.* evaluated different *T*/*C* values and found no differences by setting the response rate cut off point below 10% in contrast to below 40.[26]

As novel treatment option and to study possible predictive biomarkers we evaluated everolimus, a compound targeting the mTOR pathway. Dysregulation of the PI3K/Akt/mTOR pathway is a common event in the pathogenesis of head and neck cancer but mTOR inhibitors have not been introduced into clinical routine use in this tumor entity.[17,27] Preclinical evaluation of mTOR inhibitors in cell lines of head and neck cancer showed antiproliferative effects and induction of apoptosis.[28] Those results led to the CAPRA trial, a phase I/II trial exploring everolimus in combination with carboplatin and paclitaxel as induction regimen.[29] Temsirolimus, another mTOR inhibitor was evaluated in a phase II study design in recurrent/metastatic head and neck cancer, which showed moderate activity after failure of standard of care treatment.[30] In our experiments everolimus showed a significant growth inhibition in 20 of 29 (68%) head and neck cancer patient-

derived xenografts when given as single agent, which creates further evidence of activity of this compound in head and neck cancer. By extensive molecular profiling we aimed at identifying predictive markers for mTOR inhibition. Preclinical evaluation of activation of the PIK3/Akt/mTOR axis has been associated with response to inhibitors targeting this axis.[31] In our studies mRNA expression analysis of pathway members such as Akt1, mTOR, RPS6KB1, FKBP1B and TSC1 did not reveal a significant association between gene expression level and response to everolimus even though we observed a trend for higher RPS6KB1 expression ($p = 0.07$) within responders. Further analysis concentrated on evaluating mutational status focusing on PI3KCA, which has been reported to be associated with treatment response.[31] Within our head and neck PDX panel we observed 7 (24%) models with PI3KCA mutations, which resembles the frequency of PI3KCA mutation reported by TCGA in head and neck cancer.[14] It has been well established that PI3K mutation may lead to tumor formation and serves as predictive marker for novel inhibitors of PI3K.[17,32] We therefore explored whether mutational status was associated to everolimus response. According to Polivka *et al.* the most frequent mutation within PI3KCA is E545K,[32] which we found in three of our models (10621, 11097 and 11482). Another mutational hot spot (H1047R) is located in the kinase domain of the p110 alpha subunit, which we detected in tumor model 10110. Other less frequent mutations were detected in the model 10960 (E542K), 10924 (E542Q) and 10847(G1049R). All but one model (10621) harbouring mutations within the PI3K gene showed a significant growth inhibition (6/7,85%), when treated with everolimus but other models with PI3KCA wild-type (14/22, 63%) responded in a similar way. We therefore conclude that PI3KCA mutational status alone may not serve as a predictive marker for the stratification of patients to treatment with mTOR inhibitors. Additional biomarkers (*e.g.*, phosphoprotein assays for proteins of the pathway) or more complex combinations of biomarkers should be evaluated for their predictive power. It will be an interesting question, whether novel compounds targeting mTOR and PI3KCA together or PI3KCA alone act in dependency of the occurrence of this mutation in a heterogeneous tumors as seen in our models and in clinical routine. In conclusion, we observed a response in the majority of our PDX for the treatment with everolimus, which justifies further preclinical and clinical evaluation of this compound, and defining a predictive biomarker or a more complex biomarker pattern for patient selection remains an important goal for translational studies. Further studies using our newly established xenograft series will also concentrate on defining the best chemotherapeutic partner of everolimus and evaluation of novel targeted compounds in head and neck cancer.

Acknowledgements

The authors thank Britta Buettner, Svetlana Gromova, Ole Daberkow and Carsta Werner for outstanding technical assistance. Jens Hoffmann reports to be the owner of EPO GmbH. All other authors have no conflict of interest to declare.

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