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# EGFR mono-antibody salvage therapy for locally advanced and distant metastatic penile cancer: Clinical outcomes and genetic analysis

Kang-bo Huang, B.S.[1], Ran-yi Liu, Ph.D.[1,*], Qi-hua Peng, B.S., Zai-shang Li, M.D., Ph.D., Li-juan Jiang, M.D., Ph.D., Sheng-jie Guo, M.D., Ph.D., Qiang-hua Zhou, M.D., Ting-yu Liu, B.S., Chuang-zhong Deng, M.D., Kai Yao, M.D., Ph.D., Zi-ke Qin, M.D., Ph.D., Zhuo-wei Liu, M.D., Ph.D., Yong-hong Li, M.D., Ph.D., Hui Han, M.D., Ph.D.*, Fang-jian Zhou, M.D., Ph.D.

*Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou , China*

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## Abstract

**Purpose:** There are limited therapeutic options for patients with advanced penile squamous cell carcinoma (PSCC) after chemotherapy failure. Thus, we evaluated the feasibility of salvage treatment using the epidermal growth factor receptor (EGFR) mono-antibody nimotuzumab in chemotherapy-failed PSCC patients and explored potential response or resistance biomarkers.

**Materials and methods:** Six chemotherapy-failed PSCC patients with locally advanced disease or distant metastasis were enrolled consecutively to nimotuzumab treatment. Clinical responses and side effects were evaluated, and genetic characteristics of cancer specimens were analyzed through the next-generation sequencing of hotspot regions in cancer-related genes.

**Results:** Two of 6 patients showed partial responses, one was identified as having stable disease, while the other 3 had disease progression after nimotuzumab therapy. Side effects were all welltolerated. Genetic analysis revealed that TP53, CDKN2A, RB1, SMAD4, FLT3, and PIK3CA were the most frequently mutated genes in PSCC specimens, while altered KRAS, HRAS, EGFR, ERBB2, and FLT3 may be correlated with nimotuzumab resistance. Furthermore, 3 patients that were human papillomavirus-positive each showed clinical response or stable disease.

**Conclusions:** EGFR mono-antibody may be a potential modality for locally advanced PSCC patients after chemotherapy failure. Further large-scale clinical studies are needed to elucidate the role of human papillomavirus status and critical gene mutations in the clinical response to EGFR-targeted therapy. 

*Keywords:* EGFR mono-antibody; Penile cancer; Salvage therapy; Genetic alterations; Human papillomavirus

*Abbreviations:* PSCC, penile squamous cell carcinoma; EGFR, epidermal growth factor receptor; TIP, paclitaxel, isofamide plus cisplatin; TPF, cisplatin, paclitaxel plus fluorouracil; SCC-Ag, squamous cell carcinoma antigen; FFPE, Formalin-fixed and paraffin-embedded; HPV, human papillomavirus; PR, partial response; ORR, objective response rate; SD, stable disease; PD, progression disease; OS, overall survival; PFS, progression-free survival

## 1. Introduction

For locally advanced (including inguinal and pelvic lymph node metastatic disease) and distant metastatic penile squamous cell carcinoma (PSCC), multimodal treatments utilizing surgery, chemoradiotherapy and/or chemotherapy continue to be the most popular treatment regimen.

*Corresponding authors. Tel: +86 2087342303.
*E-mail addresses:* hanhui@sysucc.org.cn (H. Han), liury@sysucc.org.cn (R.-y. Liu).
[1]Kang-bo Huang and Ran-yi Liu contributed equally to this work.

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Although several chemotherapy regimens combined with surgery have achieved encouraging short-term efficacies, long-term survival for those patients remains poor [1,2]. For patients with chemotherapy-failed experience, therapeutic options are even more limited.

Epidermal growth factor receptor (EGFR)-targeted therapy has been reported to provide clinical benefits for PSCC patients in several studies [3,4]. Here, we evaluated the efficacy and tolerance of nimotuzumab, a humanized EGFR mono-antibody, in the salvage treatment of locally advanced and distant metastatic PSCC and analyzed the genetic basis of responses or resistance to nimotuzumab treatment.

## 2. Patients and methods

### 2.1. Patient population and tumor specimens

Between January and June 2017, 6 consecutive PSCC patients who had received radical or partial penectomy and had recurrence after receiving cisplatin-based chemotherapy were enrolled and treated with nimotuzumab in the Department of Urology, Sun Yat-sen University Cancer Center. Each patient had an Eastern Cooperative Oncology Group performance status of 0 or 1 and presented with normal hematological, renal, and liver function. They all presented inguinal lymph node metastasis and 1 patient presented with distant metastasis (lung, bone). Before admission, 2 patients had received paclitaxel, ifosfamide and cisplatin (TIP) chemotherapy, and the other 4 patients had received cisplatin, paclitaxel and fluorouracil (TPF) regimen. Detailed patient characteristics are listed in Table 1.

Patients received 400 mg nimotuzumab (Biotech Pharmaceutical Co., LTD, Beijing, China) on days 1 and 8 of a 21-day cycle until disease progression or voluntarily withdraw. Patients who were identified as having a clinical response and who also met the surgical indications received consolidated surgery (plus myocutaneous flap reconstruction if necessary), and then followed by another 2 cycles of EGFR-targeted therapy once they recovered from surgery. This study was approved by the Institutional Review Board and Human Ethics Committee of Sun Yat-sen University Cancer Center, and written informed consent was obtained from each patient.

An imaging examination was conducted before each cycle, and efficacy was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST v1.1) [5]. Progression-free survival (PFS) was measured from the regimen start date up until disease progression or death. Patients were divided into responders and non-responders, according to the evaluation of clinical response. Adverse effects were graded according to the Common Terminology Criteria for Adverse Events v3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf). Serum squamous cell carcinoma antigen levels were measured 3 to 5 days prior to each cycle. Follow-up programs began after completion of treatment and ended in June 2018.

Tumor specimens were collected before nimotuzumab treatment (after previous chemotherapy failure) and after nimotuzumab treatment during the subsequent surgery.

### 2.2. HPV status and gene mutation analysis

Formalin-fixed and paraffin-embedded (FFPE) tumor tissues (four 10-$\mu$m thick sections per sample) were deparaffinized and rehydrated, and total DNA was extracted using the FFPE DNA kit (Omega Bio-tek). Human papillomavirus (HPV) status was detected by the DaAn Clinical Laboratory Center (Guangzhou, China) using fluorescence quantitative real-time polymerase chain reaction (FQ-PCR) with the PGMY09/11 primer system as previously reported [6].

Mutation hotspot regions of 50 cancer-associated genes (Supplementary Table 1) were analyzed with next-generation sequencing by Wuxi Apptec (Shanghai, China) using the Ion AmpliSeqTM Cancer Hotspot Panel v2 (Thermo Fisher Scientific). Hotspot regions covered approximately 2,800 COSMIC mutations of 50 genes (including KRAS, BRAF, and EGFR). Variations were filtered out if the variation allele frequency was $<0.05$ or recorded in either 1000g2014oct_eas ($>0.05$, http://www.1000genomes.org) or SNP142common database (http://www.ncbi.nlm.nih.gov/SNP/).

Prioritized functional single nucleotide variations and insertion/deletions were identified if one of the following criteria was met: (1) splicing site, frameshift insertion/deletion or stopgain/ stoploss mutations; (2) nonsynonymous mutations where the functional impacts were “deleterious ” predicted by Sorting Intolerant From Tolerant (SIFT) or Polyphen2_HDIV. KEGG pathway analysis was performed and functional mutations in canonical pathways were identified with a $q$ value of less than 0.05 [7].

### 2.3. Immunohistochemistry analysis

After routine deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase inactivation, the FFPE slides (4.0-$\mu$m thick) were incubated overnight at 4°C with an EGFR antibody (clone E114, Ascend Biotechnology) at 1:100 in Dako antibody diluent (Dako). After incubating with secondary antibodies (Envision, Dako), the slides were visualized by DAB staining [8,9].

EGFR expression scores in tumor tissues were assessed based on the percentage of stained cells (1+, 6%–25%; 2+, 26%–50%; 3+, 51%–75%; and 4+, >75%) as previously reported [9]. The specimen was identified as EGFR overexpression if the score was 3+ or 4+.

## 3. Results

### 3.1. Patients and EGFR antibody treatment outcomes

Four of the 6 patients received over 3 cycles of treatment, while 2 received only 1 cycle due to disease

Table 1
Clinical characteristics and outcomes of nimotuzumab treatment for penile cancer

| | | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 |
|---|---|---|---|---|---|---|---|
| Age (years old) | | 43 | 51 | 61 | 75 | 51 | 72 |
| ECOG performance status | | 0 | 0 | 1 | 1 | 1 | 1 |
| Tumor stage | | $pT_2N_3M_0$ | $pT_2N_3M_0$ | $pT_1N_3M_0$ | $cT_2N_2M_0$ | $pT_2N_2M_1$ | $pT_xN_3M_0$ |
| Prior surgical treatment | | Total penectomy, ILND | Partial penectomy, ILND | Wide local excision, ILND, PLND | Partial penectomy | Partial penectomy, ILND | Wide local excision, ILND |
| Prior chemotherapy | | TIP | TPF | TPF | TPF | TIP | TPF |
| Site of tumor specimen | | Primary tumor (before) Inguinal mass (after) | ILNs (before) ILNs (after) | ILNs | Primary tumor | Primary tumor | ILNs |
| Recurrence type | | Regional | Regional | Regional | Regional | Regional and distant | Regional |
| HPV status | | +(HPV16) | +(HPV16) | − | +(HPV16) | − | − |
| Nimotuzumab treatment cycles | | 4 | 4 | 3 | 3 | 1 | 1 |
| Site of target lesions | | Inguinal mass | ILNs PLNs | ILNs, suprapubic skin nodules | ILNs | ILNs, PLNs, Bone, lung | ILNs |
| Best response | | CR | PR | PD | SD | PD | PD |
| Toxic effects | | Skin rash (I) | Constipation (I) | Nausea (I) | Skin rash (I) | Nausea (I), skin rash (I) | Skin rash (I) |
| Subsequent treatment | | ILND, MCFR | ILND, MCFR | None | None | None | None |
| PFS (mo) | | 7 | 5 | 1 | 13 | 1 | 2 |
| OS (mo) | | 16 | 6 | 1 | 15 | 2 | 9 |
| Survival status | | Alive | Dead | Dead (of heart attack) | Alive | Dead | Dead |
| SCC-Ag (ng/ml,1.5) | Before* | 6.2 | 5.6 | 8.0 | 4.0 | > 70 | \ |
| | After* | 0.9 | 3.1 | 46.4 | 1.1 | > 70 | \ |

CR = complete response; ECOG = Eastern Cooperative Oncology Group; HPV = human papillomavirus; ILNs = inguinal lymph nodes; ILND = inguinal lymph node dissection; LN = Lymph node; MCFR = myocutaneous flap reconstruction; OS = overall survival; PD = progressive disease; PFS = progression-free survival; PLNs = pelvic lymph nodes; PLND = pelvic lymph node dissection; PR = partial response; SCC-Ag = squamous cell carcinoma antigen; SD = stable disease; TIP = paclitaxel + ifosfamide + cisplatin; TPF = cisplatin + paclitaxel + fluorouracil.

* Before: before nimotuzumab treatment. After: after finishing all the treatment cycles of nimotuzumab treatment.

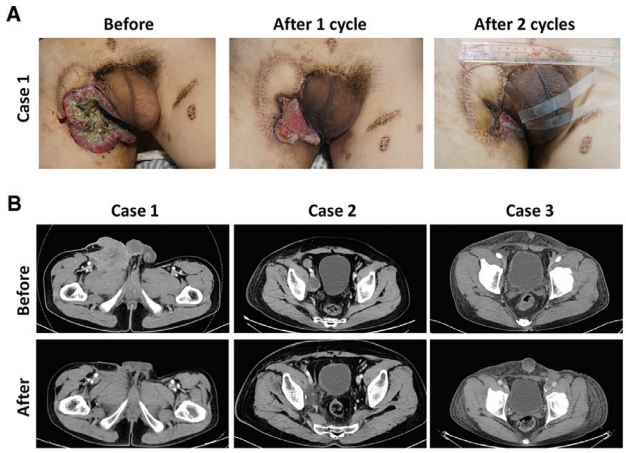


Fig. 1. Representative pictures of efficacy evaluation. (A) Macroscopic appearance of case 1 before and after nimotuzumab treatment. (B) Cross-sectional computer tomography axial images before nimotuzumab treatment and after 2 cycles of nimotuzumab treatment (cases 1–3). After 2 cycle treatments, case 1 had a marked response at the right inguinal mass which almost disappeared, case 2 presented with right metastatic pelvic lymph node necrosis, and case 3 had an enlarged subcutaneous nodule.

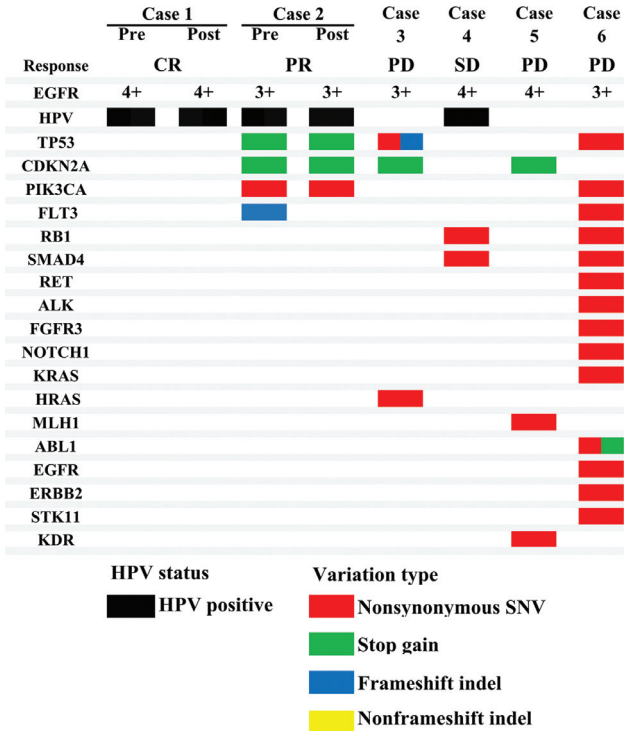


Fig. 2. Significant genetic alterations in 6 patients treated with an EGFR antibody. The heatmap shows the mutation status for selected genes in each patient; cases 1 and 2 were additionally tested after treatment.

progression. Two of 6 patients (cases 1 and 2) showed a partial response after 2 cycles (Fig. 1) with 33.3% objective response rate (ORR). Interestingly, the size of the inguinal mass in case 1 decreased to almost disappearance after 2 cycles of treatment. These 2 clinical responders received subsequent consolidated inguinal lymph node dissection along with myocutaneous flap reconstruction followed by another 2 cycles of nimotuzumab treatment. One patient (case 4) showed stable disease (SD) after 3 cycles of treatment and refused consolidated surgery due to personal reasons. The other 3 patients experienced disease progression.

This EGFR-targeted therapy was well-tolerated, and the most common side effect was a grade-I skin rash that occurred in 4 of 6 patients. Other side effects included nausea and constipation (Table 1). After a median 14 months of follow-up, 3 patients (cases 2, 5, and 6) died of penile cancer, and 1 (case 3) died of a heart attack. The average PFS and overall survival (OS) were 4.8 and 9.2 months, respectively. Squamous cell carcinoma antigen levels decreased in 3 clinical beneficiaries, including patients who showed responses or SD after treatment, but not in progression disease cases. Detailed patient and disease characteristics are shown in Table 1.

### *3.2. EGFR expression, HPV status, and genetic mutation analysis*

All tumor specimens demonstrated EGFR overexpression (3+ or 4+), and the specimens of 2 responders exhibited similar immunohistochemistry scores before and after treatment (Fig. 2, Supplementary Fig. S1). Three cases were identified as HPV-positive (HPV subtype 16, a high-risk HPV subtype; Table 1 and Fig. 2).

Gene mutation analysis revealed that variation numbers in the hotspot regions of 50 cancer-associated genes averaged to 6.3 (8.25 for nonresponders vs. 2.5 for responders; Supplemental Table S2). Interestingly, case 1 (one of the responders) showed no variation in these hotspot regions. The most frequently mutated genes were TP53, CDKN2A (in 3 cases), RB1, SMAD4, FLT3, and PIK3CA (in 2 cases; Fig. 2).

Nonresponders harbored more mutated genes that may be associated with anti-EGFR treatment resistance, including KRAS, HRAS, RB1, RET, ALK, FGFR3, NOTCH1, SMAD4, MLH1, ABL1, EGFR, ERBB2, ERBB4, STK11, and KDR mutations, than responders (Fig. 2). According to KEGG pathway analysis, these genes were mainly involved in the MAPK and ERBB pathway (Table 3). Additionally, some interesting changes in gene mutations following anti-EGFR treatment were noted in one of the responders with partial response outcomes (case 2). For example, the FLT3: NM_004119:exon16:c.1999delC:p.Q667fs mutation disappeared after treatment (Table 2), and the alteration frequencies of TP53, PIK3CA, and CDKN2A slightly decreased from 0.49 to 0.36, 0.34 to 0.26, and 0.55 to 0.36, respectively.

## 4. Discussion

A huge challenge for advanced PSCC treatment is local recurrence (mainly inguinal recurrence). Salvage surgical resection, as reported by Baumarten et al. [10], was the potential treatment modality in this setting. However, surgery alone may be not enough for achieving good clinical

Table 2
Changes in the gene mutations in paired tumor specimens of case 2 before and after nimotuzumab treatment

| Specimen source | Median depth | Somatic short variants | Alteration frequency |
|---|---|---|---|
| Pre- | 1147 | FLT3:NM_004119:exon16:c.1999delC:p.Q667fs | 0.96 |
| Post- | 8355 | No mutation at FLT3:NM_004119:exon16:c.1999 | 0 |
| Pre- | 1301 | TP53:NM_001126115:exon2:c.C190T:p.R64X | 0.49 |
| Post- | 9321 | TP53:NM_001126115:exon2:c.C190T:p.R64X | 0.36 |
| Pre- | 1164 | CDKN2A:NM_000077:exon2:c.C238T:p.R80X | 0.55 |
| Post- | 6591 | CDKN2A:NM_000077:exon2:c.C238T:p.R80X | 0.36 |
| Pre- | 927 | PIK3CA:NM_006218:exon10:c.G1633A:p.E545K | 0.34 |
| Post- | 6007 | PIK3CA:NM_006218:exon10:c.G1633A:p.E545K | 0.26 |

Table 3
Functional mutated genes in significantly mutated KEGG pathways

| | Case1 | | Case2 | | | | | |
|---|---|---|---|---|---|---|---|---|
| Signaling pathway | Before | After | Before | After | Case3 | Case4 | Case5 | Case6 |
| MAPK | None | None | None | None | HRAS | None | None | KRAS, FGFR3, EGFR |
| ERBB | None | None | PIK3CA | PIK3CA | HRAS | None | None | KRAS, PIK3CA, EGFR, ERBB2, ABL1 |
| Wnt | None | None | None | None | APC | None | None | None |
| p53 | None | None | TP53 CDKN2A | TP53 CDKN2A | TP53 CDKN2A | None | CDKN2A | TP53 |
| mTOR | None | None | PIK3CA | PIK3CA | None | None | None | STK11, PIK3CA |

outcome [1,11,12]. Multimodal treatment including surgery, chemoradiotherapy and/or chemotherapy was usually needed for recurrent PSCC. When this disease develops into distant metastasis, surgery and chemoradiotherapy can only be used for palliative local disease control and palliative systemic chemotherapy should be considered.

Disappointedly, chemotherapy efficacy for advanced PSCC is far from satisfactory. Although cisplatin-based chemotherapy is the standard regimen, ORR is only approximately 30% to 40%, and PFS is relatively short [13]. Among cisplatin-based regimens, traditional bleomycin, methotrexate, and cisplatin regimen have rarely been used due to prohibitive toxicity [13]. Neoadjuvant TIP and cisplatin plus fluorouracil regimens showed ORRs of 50% and 32%, respectively, and PFS times of 8.1 and 5 months, respectively [1,2]. For cisplatin-based chemotherapy-failed patients, subsequent treatment choices are more restricted. Single-agent paclitaxel achieved a 20% ORR and a 2.75-month PFS [14]. As taxanes are generally used in first-line treatment regimens, there are limited treatment options following failure of cisplatin and taxanes.

The binding of a ligand to EGFR activates several intracellular signal transduction pathways which stimulate cell proliferation and neoangiogenesis while inhibiting apoptosis [15]. Inhibitors of EGFR, anti-EGFR antibodies, or EGFR tyrosine kinase have been used with promising results. Carthon et al. [3] reported the first retrospective study concerning single-agent EGFR-targeted therapy (cetuximab, erlotinib, or gefitinib) or therapy combined with chemotherapy in locally advanced (12 cases) or distant metastatic PSCC patients (12 cases). Most of these patients (91.7%) had received chemotherapy previously. After treatment, 7 patients responded, including 4 with locally advanced disease and 3 with distant metastases. The average PFS and OS times were 2.8 and 7.4 months, respectively, and the ORR was approximately 25%. In another retrospective study by Necchi et al. [4], 6 of 10 chemotherapy-failed patients obtained a clinical response to panitumumab treatment. The median PFS and OS times were 1.9 and 9.5 months, respectively. However, no patients with distant metastasis (0/6) showed a clinical response. Recently, in a phase-2 clinical study, an EGFR tyrosine kinase inhibitor, dacomitinib, was used as a first-line agent for locally advanced or metastatic PSCC and obtained a 32.1% ORR and a 4.1-month median PFS time, but only 1 of the 8 patients with distant metastasis responded to dacomitinib [16]. These clinical studies provide preliminary evidence for EGFR-targeted therapy. In addition, there is a phenomenon that patients with distant metastases seem to be less sensitive to treatment than patients with locally advanced disease.

In our study using the EGFR mono-antibody nimotuzumab as a salvage treatment regimen in EGFR-overexpressed and advanced PSCC patients, the toxicities were all tolerable, and 2 of 5 locally advanced patients showed remission while 1 displayed SD. It appears that part of regional recurrent patients benefitted from EGFR-targeted therapy. This phenomenon is in accordance with previous reports, as mentioned above [3,4,16]. However, it is

unknown whether PSCC patients with distant metastasis benefit from this treatment as there was only 1 case with distant metastasis in this study. Besides, the patient who showed SD (case 4) without consolidated surgery remained alive by the time of last follow-up, it is reasonable to speculate that both EGFR-targeted therapy and low-grade malignant biology of tumor contribute to his survival.

Although EGFR overexpression is common in PSCC (up to 92%) [17], some patients with EGFR overexpression were found to be resistant to EGFR-targeted therapy. Additionally, a part of patients without EGFR overexpression were found to respond to this therapy (25% ORR) [17,18]. In this study, all 6 patients showed EGFR overexpression (3+ or 4+) regardless of responding to treatment. Thus, it is necessary to finding a biomarker that can predict a clinical response and resistance.

The most important signal transduction pathways involved in the activation of EGFR are the RAS/MAPK and phosphatidylinositol-3-kinase (PI3K)/Akt pathways [15]. In a previous dacomitinib clinical trial [16], genetic analysis revealed that telomerase reverse transcriptase gene mutations and PI3K/mTOR pathway-involved genes may correlate with EGFR-TKI resistance. Our genetic analysis also found that nonresponders harbored several significant mutated genes which possibly affected their EGFR treatment outcome, including KRAS, HRAS, EGFR, and ERBB2. These genes are heavily involved in the MAPK and ERBB pathways. This suggests that the mechanism of resistance for such patients may be a result of abnormal activation, enhanced by those mutations, of the RAS/MAPK signaling pathway.

KRAS mutations are regarded as one of the most common alterations corresponding to the resistance of anti-EGFR treatment in colorectal and other cancers [19,20]. HRAS mutations were found to enhance drug resistance and appear less frequently than KRAS mutations in many cancers [21]. Although these mutations are considered to be rare in PSCC cases [17,22,23], a KRAS mutation and an HRAS mutation were found in 2 nimotuzumab-resistant patients (cases 6 and 3, respectively) in this study. Other mutated genes including EGFR and ERBB2 have been previously reported to correlated with EGFR-targeted treatment resistance in colorectal cancer [19,24], and were also found in nonresponders in this study. These mutations may contribute to the observed resistance of patients to nimotuzumab treatment.

Amplification or activated mutation of *FLT3* have been reported to confer resistance to EGFR inhibitors [25]. Surprisingly, a frameshift mutation of FLT3/Q667fs was found in case 2 and disappeared after nimotuzumab treatment. It is unclear whether this phenomenon correlates with nimotuzumab sensitivity, but we hypothesize that an FLT3/Q667fs mutant loses its tyrosine kinase function thereby diminishing its resistance to EGFR-targeted therapy. This mechanism will require verification with future research.

Previous reports estimated that approximately 33% to 80% of penile cancers were related to HPV, especially the high-risk types HPV16 and 18 [9,26,27]. The presence of high-risk HPV DNA has been reported to confer a survival advantage for PSCC [28]. Our EGFR-targeted therapy study yielded an interesting result: 3 clinical beneficiaries were positive for HPV subtype 16 and other 3 HPV-negative patients were all resistant to anti-EGFR treatment. In fact, several studies support our observation: HPV16 E5 protein was found to up-regulate EGFR-mediated signal transduction, indicating that HPV-positive patients may have more active EGFR pathways [29]. Furthermore, HPV infection rates in laryngeal squamous cell carcinoma were significantly higher in EGFR overexpression cases ($T = 440$; $P = 0.002$) [30]. The correlation between HPV status and the EGFR mono-antibody response needs further investigation.

There were several limitations to this study. First, the rarity of PSCC contributed to the small sample size of this study, and it was hard for us to perform statistical analysis and further draw a firm conclusion. Thus, we can only propose several hypotheses with our limited experience. Second, the heterogeneity of the patients involved in this study was notable. Two of the responders were younger than the others; the patients' prior chemotherapy regimens were not exactly the same (TPF or TIP), though all were cisplatin-based regimens. These limitations could affect the results of this study.

Although limited by the small sample size, our study suggested the promising efficacy and tolerability for EGFR-antibody nimotuzumab treatment as an alternative salvage treatment for advanced penile cancer. HPV infection, along with EGFR, KRAS, HRAS, ERBB2, and FLT3 mutations, may be associated with EGFR antibody resistance/sensitivity in PSCC. To further verify our hypotheses, we plan on registering a phase-II clinical trial of anti-EGFR therapeutics in patients with advanced PSCC.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.urolonc.2018.10.016.

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