Curr Probl Cancer 39 (2015) 137–145

Contents lists available at ScienceDirect

Curr Probl Cancer

journal homepage: www.elsevier.com/locate/cpcancer

Molecular markers in penile cancer

Simon Rodney, MRCS, MSc, MA, Andrew Feber, PhD, Manit Arya, MD, FRCS (Urol), Asif Muneer, MD, FRCS (Urol)

Introduction

In Europe and North America penile cancer is a rare disease, with an incidence of approximately 1.5 per 100,000 males, although this increases to 4.4 per 100,000 men in South America and Africa. Penile cancer represents a significant global health problem due to the often devastating consequences of treatment at this site of malignancy, and the mortality associated with metastatic disease.[1]

The primary lymphatic drainage of penile cancer is to the inguinal lymph nodes, and the presence of metastatic disease within the inguinal lymph nodes is the most important prognostic factor.[2] The 5-year survival for men with lymph node metastasis is 57%, compared with 90% for those without.[3] Of those patients who present with clinically node negative (cN0) disease, 20%-25% will have occult metastases.[4] Therefore if all patients with $\geq$T1G2 disease undergo inguinal lymphadenectomy, potential overtreatment will occur in 75%-80% of cases where the inguinal lymph nodes are pathologically clear. Furthermore, open inguinal lymphadenectomy is associated with significant morbidity, with up to 70% of patients developing complications related to wound healing, or long-term genital or lower limb lymphoedema.[5] Therefore, there is a clear clinical need to accurately predict the presence of lymph node micro-metastasis and to determine prognosis so as to select only those patients who would benefit from radical inguinal lymphadenectomy. This information would be invaluable when discussing and determining the extent of surgical resections and further treatment required.

This review will evaluate the evidence for the use of molecular biomarkers to predict lymph node status as well as prognosis in penile carcinoma. It will also discuss the next generation of biomarkers, which have the potential to change the diagnostic landscape in penile cancer. The pathways and logic behind biomarkers that have been studied thus far will also be considered.

Current molecular knowledge

Most of the proposed biomarkers are based on knowledge of the oncogenic pathway of all malignancies and specific penile cancer molecular pathways.

http://dx.doi.org/10.1016/j.currproblcancer.2015.03.005

For a cancer cell to become malignant it needs to accomplish several steps, including the following:

1. lose DNA repair and cell cycle control mechanisms,
2. subvert growth signaling pathways,
3. gain a blood supply,
4. develop the ability to invade other tissues.

The biomarkers examined are therefore based on one of these steps using genomic, proteomic, epigenomic, and expression detection mechanisms. Overexpressed biomarkers that play a vital role in the oncogenesis of the disease are especially valuable as they may be not only useful for prognosticating but also serve as a chemotherapeutic target. This review will use published studies on biomarkers used for the detection, prognosis, and surveillance of penile cancer. Furthermore, the exciting potential of biomarkers currently in development using next-generation sequencing techniques will also be discussed.

A table with a summary of all the biomarkers that will be considered is shown in Fig 1 together with the relationship to lymph node status and disease-specific mortality.

DNA repair and cell cycle control mechanisms

One of the main processes by which cancer develops is by progressive disruption of the normal anti-proliferative cell control systems. The proliferation of a cell is highly regulated to ensure genetic stability. A healthy cell will enter a cell replication cycle only after receiving external growth factors that activate mitogen receptors. These factors can then signal through transduction mechanisms using tyrosine kinases to activate G1 and G1/S cyclin-dependent kinases to drive entry into the cell cycle. Various control points exist that guard entry into the cell cycle before DNA replication. One of the most important control mechanisms ensures that the cell will not replicate in the presence of DNA damage. The tumor suppressor gene p53 is one of the genes most characterized for its role in ensuring genetic stability; p53 is a transcription factor that can either delay cell division or activate apoptosis, depending upon the extent of DNA damage; p21 is a cyclin-dependent kinase inhibitor that can be induced both by p53, and independently, in response to stress and DNA damage. Its induction results in cell cycle arrest, preventing tumor development. Mutations in both of these tumor suppressor genes are among the most commonly found in mammalian cancers.

Several studies have been undertaken to examine the association between p53 expression and immunoreactivity with lymph node metastases and prognosis. Significant positive associations between p53 immunoreactivity and disease-specific mortality in multivariate analyses have been reported in 5 studies.[6-10] Gunia et al[7] reported a hazard ratio (HR) of 3.2 ($P = 0.041$) for disease-specific mortality in p53-positive tumors in 110 patients. Lopes et al[10] found that patients with tumors that stained positive for p53 had a worse 10-year survival rate (26.4%) than those whose tumors stained negative (54.6%) ($P = 0.009$) and also found a positive association with lymph node metastases with a relative risk of 4.8 (95% CI = 1.6-14.9) for lymph node positivity in p53-positive tumors. This finding was corroborated also in 2 other studies with a relative risk of 6.01 (95% CI = 1.402-25.764) in a group of 73 Chinese patients.[6] Many of these studies have large CIs, signifying uncertainty in the results owing to small sample sizes. Cyclin D1 and p21 were not significantly associated with disease-specific mortality in a multivariate analysis of 110 patients.[7]

$p16^{INK4a}$ inhibits G1 cyclin-dependent kinases 4 and 6, and the cyclin D-dependent kinases, which initiate phosphorylation of the retinoblastoma tumor suppressor protein Rb. $p16^{INK4a}$ therefore has the capacity to arrest cells in the G1 phase of the cell cycle in response to specific circumstances. This arrest can be permanent in response to DNA damage, an example of an important protective mechanism against genetic instability and subsequent cancer development. In many cancers this pathway is dysregulated.[11] The status of $p16^{INK4a}$ as a prognosticator has been examined by assessing its expression relative to cancer-specific survival. Gunia et al reported $p16^{INK4a}$ expression levels in 92 patients with invasive penile cancer treated with either partial or total penectomy. Multivariate analysis identified $p16^{INK4a}$ as a marker for favorable

Biomarker	Lymph node status	Disease specific mortality
p53	Positive correlation	Predicts poor survival
	Relative risk 4.8 (95% CI = 1.6-14.9)[6]	Hazards ratio 3.2 p=0.041[7]
p21	Not tested	No statistical association with multivariate analysis
Cyclin D1	Not tested	No statistical association with multivariate analysis
$p16^{INK4a}$	Not tested	Predicts worse survival
		hazard ratio of 0.025 p=0.011[8]
MCM2	No statistical association	No statistical association with multivariate analysis
Ki-67	Generally no statistical association	No statistical association with multivariate analysis
KAI1	Unclear	Further evidence needed to demonstrate.
D2-40	Positive association	Not tested
	Sensitivity = 83.3% specificity= 78%[9]	
E-cadherin	Unclear	No statistical association
CD147/Extracellular matrix metalloproteinase inducer (EMMPRIN)	Not tested	5 year survival relative risk = 420 (95% CI= 51-3460) [10]
MMP-2	No statistical association	No statistical association
MMP-9	No statistical association	No statistical association
ANX AI	Possible association at invasion front p=0.001[11]	No statistical association
ANX AII	No statistical association	No statistical association
Periostin	Not tested	Predicts hazard ratio 1.44 (1.14-1.81) [12]
CD44	Small association p=0.03[13]	Not clear
SCCA	Positive association but very small sample sizes	Negatively associated with disease free survival
	Sensitivity = 57% Specificity = 100%[14]	Odds ratio 0.13 p=0.006 (0.034-0.55) [15]
HPV	Unclear	Unclear
Ploidy statu	Unclear	Strong statistical association HR 4.19, 95% CI = 1.17-14.95, p=0.03)[16]
Methylation panel inc CDO1, AR1, WT1	Positively associated with lymph node metastases	Not tested
	Sensitivity = 93% Specificity = 80%[17]	Not tested

Fig. 1. Table summarizing the associations of biomarkers with lymph node status and disease-specific mortality.

prognosis with a HR of 0.44 (95% CI = 0.23-0.84, $P = 0.013$).[12] The 5-year cancer-specific survival was 85% in patients with $p16^{INK4a}$ expression but fell to 57% for those without.

Each replicating cell uses a DNA replication-licensing pathway to control the proliferative state of cells and to ensure that DNA is replicated only once per cell cycle. This is important to ensure genomic

stability. Activation of oncogenes can disrupt this regulatory pathway. MCM2 is expressed throughout the cell cycle but is down-regulated during quiescence or senescent states. Geminin is also involved in cell cycle control and is an inhibitor of DNA replication. It accumulates during the S, G2, and M phases of the cell cycle and is degraded at the metaphase-anaphase transition, permitting replication in the succeeding cell cycle. Geminin and MCM proteins can therefore represent biomarkers for cell cycle control and are implicated in oncogenesis.[13] The use of MCM2 has been tested as a biomarker in 2 penile cancer studies. May et al[14] examined the association between expression of MCM2 and disease-specific mortality in 158 patients but found no statistically significant relationship. However, Kayes et al[15] examined MCM2 with respect to lymph node metastasis and found a positive association ($P = 0.02$) in univariate analysis, but this was no longer significant when multivariate analysis took into account other clinical and pathological parameters. Malfunction of the DNA-replicating machinery can result in DNA ploidy. Totally 141 samples of penile carcinoma were examined for the presence of aneuploidy. Univariate analysis demonstrated that aneuploidy is a strong, independent prognosticator for overall survival (HR = 4.19, 95% CI = 1.17-14.95, $P = 0.03$).[15]

Ki-67 is a reliable way of evaluating tumor cell proliferation as it is a nonhistone nuclear matrix protein which is expressed in all phases of the cell cycle except G0.[16] The relationship of Ki-67 expression with disease-specific mortality and lymph node metastases was examined in 5 studies. All studies were negative for the association with disease-specific mortality[6,14,17,18] and only 1 found an association with lymph node metastases; immunohistochemical expression of Ki-67 in 125 patients found the relative risk for lymph node metastases and on multivariate analysis to be significant at 3.73 (95% CI = 1.4-9.7).[17]

Subversion of cell-signaling pathways

Dysregulation of the signaling pathways of growth receptors with tyrosine kinase activity is well established in various cancers. These growth receptors control several signaling pathways, including PI3K and the Ras pathway. PI3K pathway exerts its activity on a large number of downstream targets, including cell proliferation, motility, adhesion, growth, and trafficking. Within this pathway PTEN acts as a negative regulator, thus functioning as a tumor suppressor. Mutations in this pathway, including PIK3CA and PTEN, have been demonstrated in penile cancer.[19] The Ras kinase pathway is also activated by receptor tyrosine kinases and consists of HRAS, KRAS, and NRAS. Ras is activated by receptor tyrosine kinase, ultimately activating ERK, which regulates transcription factors controlling cell growth, differentiation, and survival.[19] Mutations in HRAS and KRAS have also been demonstrated to occur commonly within penile cancer. Interestingly, mutually exclusive mutations have been found within these 2 pathways,[19] suggesting that either pathway is sufficient for the development of penile cancer.

KAI1 is a cell membrane protein that has a role in signal transduction, cell activation, proliferation, and motility.[20] Downregulation has been associated with metastases in several carcinomas. It was originally described as a metastasis suppressor gene in prostate cancer and is associated with poor differentiation in cervical carcinomas.[20] It is therefore an intriguing candidate for providing further prognostic information in penile cancer. Loss of heterozygosity of the KAI/CD82 gene locus was not found in this penile cancer series, so alternative methods of reduced expression need to be considered.[21] KAI1 expression has previously been shown in 30 patients to have a positive association with both an increase in disease-specific mortality ($P = 0.0042$) and lymph node metastases ($P = 0.0002$).[21]

Angiogenesis and invasion

Angiogenesis and lymphangiogenesis are required for invasion and spread to inguinal lymph nodes. The antibody D2-40, an epitope on the podaplanin antigen expressed in lymphatic endothelial cells, enables identification of the location and density of lymphatic vessels. Podoplanin expression is upregulated in several squamous cell carcinomas, and could play a role

in penile cancer oncogenesis and in the prediction of lymph node status.[22] D2-40 expression was measured in 39 patients with penile cancer, with a follow-up of 100 months. On multivariate analysis no statistical difference was found in disease-specific mortality based on high or low D2-40 expression levels. However, D2-40 expression with intratumoral lymphatic vessel density of greater than 2 had a sensitivity of 83.3% and a specificity of 78%.[22]

E-cadherins are cell-to-cell adhesion molecules that play a vital role in limiting proliferation. They are essential in maintaining tissue morphogenesis and epithelial integrity and therefore play an important role in limiting invasive potential of cells.[23] Low levels of E-cadherin immunoreactivity have been associated with increased risk of metastases in several malignancies. E-cadherin as a biomarker for penile cancer was evaluated in 2 studies. Campos et al[24] evaluated E-cadherin with respect to recurrence in 125 patients and found low levels to be associated with lymph node metastases. Zhu et al[6] evaluated expression levels in 73 patients and found no association with either lymph node metastases or disease-specific mortality.

CD147 or extracellular matrix metalloproteinase inducer overexpression has been associated with invasiveness and metastatic potential. It induces production of matrix metalloproteinases (MMPs) in the surrounding tumor and mesenchymal cells. This results in degradation of the extracellular matrix, facilitating invasion of tumor cells and angiogenesis.[25] High expression levels have been identified in other tumors such as breast and lung carcinomas. An analysis for CD147 expression in tumor samples from 17 patients revealed a significant positive association for 5-year survival, with a relative risk of 420 (95% CI = 51-3460).[25] The extremely large confidence intervals reflect uncertainty in the results owing to the small sample size, but given the strongly positive result, this biomarker should be considered for further analysis in a larger cohort. Expression levels of MMP-2 and MMP-9 have been considered in 2 studies. Kato et al examined 125 patients with penile squamous cell carcinoma[24] and reported no association with lymph node metastases but the penile carcinoma recurrence rate in patients with and without MMP-9 expression was significantly increased with a relative risk of 3.2 (95% CI = 1.28-8.3).[24] Zhu et al[6] studied 73 patients and similarly found no association with prognosis.

Annexins are a group of calcium- and phospholipid-binding proteins that have particular roles in signal transduction, proliferation, and migration. They are involved in the invasion of tumor cells. Annexin I (ANX A1) is involved in cell proliferation and migration, whereas ANX AII plays a role in DNA synthesis and RNA binding.[26] ANX AI and ANX AII expression levels were assessed in 29 patients with invasive penile squamous cell carcinoma. No association was found with disease-specific survival with a follow-up for a minimum of 2 years. However, at the invasion front, ANX AI but not ANX AII was associated with lymph node metastases ($P = 0.001$).[26]

Periostin is a secreted cell adhesion molecule implicated in invasion of breast, ovarian, lung, and esophageal carcinomas by altering the local tissue microenvironment, possibly through activation of the PI3K pathway. In a study 89 patients with penile carcinoma, periostin expression was associated with a reduced cancer-specific survival with a HR of 1.44 (95% CI = 1.14-1.81) and $P = 0.002$.[27]

Other potential biomarkers

CD44 is a cell membrane protein that can serve as a marker for tumor-initiating cells or cancer stem cells. In a sample of 39 patients with nonpalpable inguinal lymph nodes, 73% with lymph node involvement demonstrated high CD44 expression levels compared with 44% with negative lymph ($P = 0.03$).[28] This marker is not specific enough on its own but it may be useful in a combined panel with other biomarkers.

Squamous cell carcinoma antigen (SCCA) is an endogenous serine protease inhibitor originally identified in the serum of patients with squamous cell carcinoma of the uterine cervix.[29] Since then elevated levels of SCCA have been used as a biomarker in multiple carcinomas, including those of cervix, lung, and liver. Several studies have examined serum SCCA levels in patients with penile carcinoma.[30-32] Laniado et al[30] examined only 11 patients but calculated a sensitivity of 57% (95% CI = 18-90) with specificity of 100% (95% CI = 40-100), the

large CI reflecting the small sample size. Touloupidis et al[31] studied a larger cohort of 63 patients and although the test did not detect occult metastases it did predict palpable nodes ($P = 0.005$). At levels of SCCA above 1.5 μg/L the sensitivity was 34.3% and specificity 89.3%. SCCA was associated with reduced disease-free survival with an odds ratio of 0.13 (95% CI = 0.034-0.55). Further work is needed to improve the diagnostic accuracy of SCCA as a clinical serum-based marker.

Human papillomavirus (HPV) is a DNA virus that replicates in the epithelium of the urogenital tract, upper respiratory tract, and the skin.[33] The many subtypes can be broadly divided into high-risk (ex/16 and 18) and low-risk HPV subtypes (ex/6 and 11). The high-risk subtypes are responsible for carcinogenesis in cervix, head, and neck, as well as anal and penile carcinomas. Low-risk subtypes are responsible for benign diseases such as anogenital warts. HPV encodes proteins E6 and E7, which can bring about genetic instability and oncogenesis. Fig 2 demonstrates the major oncogenic effects of proteins E6 and E7. The prevalence of HPV DNA within penile carcinoma ranges between 20% and 80%.[35-37] Several retrospective studies have been undertaken with varying survival outcomes. Ghittoni et al examined 171 tumor specimens and found a significant 5-year survival advantage with a HR of 0.14 (95% CI = 0.03-0.63).[38] This may reflect the different molecular pathway of HPV-related disease.

Deficiencies in previous biomarker research studies

Cancer Research UK has devised a useful roadmap to aid in the planning for development of diagnostic biomarkers. It details 3 stages within biomarker discovery and assay development[39]:

1. Develop an accurate and reproducible assay to measure a biomarker. Define the biomarker distribution using the assay on adequate specimens representative of the target patient population.
2. Study relationships between the biomarker and gold standard diagnostic practice using clinical samples collected retrospectively.
3. Develop the biomarker assay to appropriate standards to enable the assay to be fit for clinical use.

All the studies investigating penile cancer biomarkers are early pilots at stage 1 or 2 of biomarker development. Very few have been adequately powered, and they are generally not validated independently. Furthermore, the studies lack long-term follow-up necessary to adequately calculate disease-specific outcomes. In multivariate analyses, many of the significant associations seen in univariate analysis are no longer present. This is due to the strong relationship between the biomarkers and tumor stage and grade, making it difficult to distinguish their independent effects.

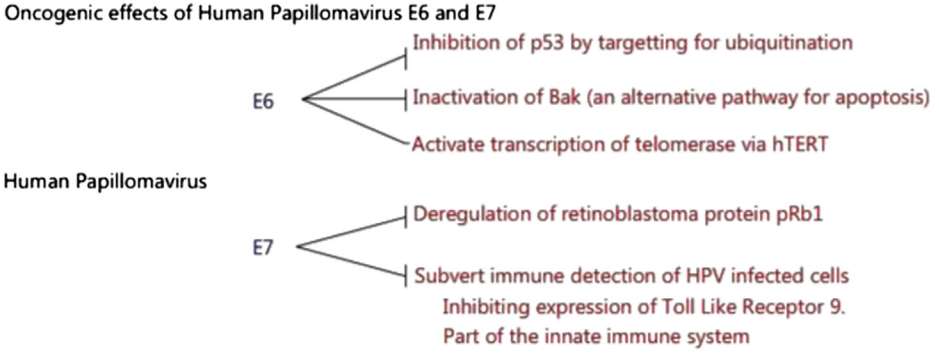

Fig. 2. Diagram demonstrating the oncogenic effects of the HPV proteins E6 and E7.[34] (Color version of figure is available online.)

The success of these biomarkers could potentially be improved by integrating further information into the test. This could include integration of additional biomarkers, clinical information, and imaging modalities. An on-going clinical trial may provide further insight into the role that molecular imaging plays in the detection of lymph node metastases in penile squamous cell carcinoma. The study will evaluate the best predictors of lymph node metastases in penile squamous carcinoma comparing magnetic resonance imaging-positron emission tomography, other imaging modalities, and sentinel lymph node biopsy (ClinicalTrials.gov Identifier: NCT02104063).

Future biomarker development

Owing to advances in the availability, cost, and speed of next-generation sequencing over the past decade, an alternative method for biomarker development is now possible. Instead of searching for proteins, tumor suppressor genes, or oncogenes, which are hypothesized to be involved in oncogenesis, the entire genome of a tumor cell can be sequenced. This will enable the discovery of new biomarkers, which can be associated with lymph node metastases or risk of progression. This technology enables large numbers of biomarkers to be selected and used together in larger panels, resulting in the increased sensitivity required for clinical purposes.

Whole exome sequencing can be employed to detect individual base pair changes in DNA sequences. Alternatively, epigenetic analysis would measure changes in methylation, histone modification, or small RNAs, which can also result in dysregulation of cell control mechanisms, leading to genetic instability and oncogenesis.[40] These epigenetic events are also associated with tumor development and provide an alternative source of biomarker discovery.

One example of this type of research is a methlylation analysis of penile cancer to discover epigenetic markers of metastasis.[40] Methylation of cytosine DNA residues is a method of controlling gene expression, and changes in DNA methylation can result in genetic instability. These changes can therefore be used as a source of tumor biomarkers. In samples from 50 patients, the authors found 6933 positions where differential methylation had occurred when comparing tumors to control samples. It was found that hypermethylation occurred in 997 regions in the tumors. Furthermore, these regions correspond to tumor suppressor genes, including CDO1, AR1, and WT1. A 4-gene epigenetic signature was identified, which could accurately predict lymph node metastasis in an independent cohort with an area under the curve (AUC) of 89%. This corresponded to a sensitivity of 93% and a specificity of 80%. More work is required to further test this marker in a larger independent cohort. If these results are validated then this assay could represent the first diagnostic test with sufficient sensitivity and specificity to replace either dynamic sentinel lymph node biopsy or superficial modified inguinal lymphadenectomy in penile cancer cases with clinically node negative disease.

Conclusions

A large number of molecular biomarkers have been tested worldwide, generally selected owing to the potential role they play in oncogenesis of penile cancer. The studies have generally been small, retrospective, and not involved matched cohorts. However, several markers have been associated positively with prognosis. The following were all associated with disease-specific mortality:

p53: HR of 3.2 ($P = 0.041$),

$p16^{INK4a}$: 27% increased survival at 5 years,

CD147 ($n = 17$); relative risk 420 (95% CI = 51-3460),

KAI1: worse 5-year prognosis ($P = 0.0042$).

Further work is needed to determine the significance of these various markers in multivariate regression models and decide what role they play in clinical practice. The following markers,

however, were all statistically nonsignificant in predicting 5-year mortality on multivariate analysis: Annexins, Cyclin D1, Ki-67, MMP-9, and PCNA.

No biomarker has yet been validated in the selection of patients for lymph node dissection. The best performer in this regard is p53 with a relative risk of 4.8 (95% CI = 1.6-14.9). D2-40 expression, a surrogate marker for lymphatic vessel density, was tested in just 39 specimens but had promising receiver operating characteristic with a sensitivity of 83.3% and specificity of 78%. Ki-67 was generally not associated with lymph node metastases, nor were MMP-2, MMP-9, or E-cadherin.

Further work should be undertaken to validate the most sensitive and specific of the biomarkers. Combinations of markers and integration with additional clinical or radiological information may improve the clinical use. If a biomarker panel can be demonstrated to have similar negative predictive values as those of sentinel lymph node dissection then it could function to stratify patients according to risk and enable frank discussions regarding oncological surgical dissection.

Further research into next-generation sequencing technologies provides hope for the creation of a clinically useful biomarker panel.

References

1. Arya M, Kalsi J, Kelly J, Muneer A. Malignant and premalignant lesions of the penis. *Br Med J* 2013;346(mar06_1): f1149. http://dx.doi.org/10.1136/bmj.f1149.
2. Ficarra V, Akduman B, Bouchot O, Palou J, Tobias-Machado M. Prognostic factors in penile cancer. *Urology* 2010;76(2 suppl 1):S66–S73. http://dx.doi.org/10.1016/j.urology.2010.04.008.
3. Moses KA, Winer A, Sfakianos JP, et al. Contemporary management of penile cancer: greater than 15 year MSKCC experience. *Can J Urol* 2014;21(2):7201–7206. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4155742&tool=pmcentrez&rendertype=abstract. Accessed 25.02.15.
4. Ornellas AA, Kinchin EW, Nóbrega BLB, Wisnescky A, Koifman N, Quirino R. Surgical treatment of invasive squamous cell carcinoma of the penis: Brazilian National Cancer Institute long-term experience. *J Surg Oncol* 2008;97(6): 487–495. http://dx.doi.org/10.1002/jso.20980.
5. Bevan-Thomas R, Slaton JW, Pettaway CA. Contemporary morbidity from lymphadenectomy for penile squamous cell carcinoma: the M.D. Anderson Cancer Center Experience. *J Urol* 2002;167(4):1638–1642. http://www.ncbi.nlm.nih.gov/pubmed/11912379. Accessed 25.02.15.
6. Zhu Y, Zhou XY, Yao XD, Dai B, Ye DW. The prognostic significance of p53, Ki-67, epithelial cadherin and matrix metalloproteinase-9 in penile squamous cell carcinoma treated with surgery. *BJU Int* 2007;100(1): 204–208. http://dx.doi.org/10.1111/j.1464-410X.2007.06908.x.
7. Gunia S, Kakies C, Erbersdobler A, Hakenberg OW, Koch S, May M. Expression of p53, p21 and cyclin D1 in penile cancer: p53 predicts poor prognosis. *J Clin Pathol* 2012;65(3):232–236. http://dx.doi.org/10.1136/jclinpath-2011-200429.
8. Martins ACP, Faria SM, Cologna AJ, Suaid HJ, Tucci S. Immunoexpression of p53 protein and proliferating cell nuclear antigen in penile carcinoma. *J Urol* 2002;167(1):89–92. [discussion 92-3]. http://www.ncbi.nlm.nih.gov/pubmed/11743282. Accessed 08.02.15.
9. Cubilla AL, Lloveras B, Alejo M, et al. Value of p16(INK)^4(a) in the pathology of invasive penile squamous cell carcinomas: a report of 202 cases. *Am J Surg Pathol* 2011;35(2):253–261. http://dx.doi.org/10.1097/PAS.0b013e318203cdba.
10. Lopes A, Bezerra ALR, Pinto CAL, Serrano SV, de MellO CA, Villa LL. p53 as a new prognostic factor for lymph node metastasis in penile carcinoma: analysis of 82 patients treated with amputation and bilateral lymphadenectomy. *J Urol* 2002;168(1):81–86. http://www.ncbi.nlm.nih.gov/pubmed/12050497. Accessed 08.02.15.
11. Ohtani N, Yamakoshi K, Takahashi A, Hara E. The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *J Med Invest* 2004;51(3-4):146–153. http://www.ncbi.nlm.nih.gov/pubmed/15460900. Accessed 28.01.15.
12. Gunia S, Erbersdobler A, Hakenberg OW, Koch S, May M. p16(INK4a) is a marker of good prognosis for primary invasive penile squamous cell carcinoma: a multi-institutional study. *J Urol* 2012;187(3):899–907. http://dx.doi.org/10.1016/j.juro.2011.10.149.
13. McGarry TJ, Kirschner MW. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* 1998;93(6): 1043–1053. http://www.ncbi.nlm.nih.gov/pubmed/9635433. Accessed 03.02.15.
14. May M, Burger M, Otto W, et al. Ki-67, mini-chromosome maintenance 2 protein (MCM2) and geminin have no independent prognostic relevance for cancer-specific survival in surgically treated squamous cell carcinoma of the penis. *BJU Int* 2013;112(4):E383–E390. http://dx.doi.org/10.1111/j.1464-410X.2012.11735.x.
15. Kayes OJ, Loddo M, Patel N, et al. DNA replication licensing factors and aneuploidy are linked to tumor cell cycle state and clinical outcome in penile carcinoma. *Clin Cancer Res* 2009;15(23):7335–7344. http://dx.doi.org/10.1158/1078-0432.CCR-09-0882.
16. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;182(3): 311–322. http://dx.doi.org/10.1002/(SICI)1097-4652(200003)182:3 < 311::AID-JCP1 > 3.0.CO;2-9.

17. Guimarães GC, Leal ML, de O, et al. Do proliferating cell nuclear antigen and MIB-1/Ki-67 have prognostic value in penile squamous cell carcinoma? *Urology* 2007;70(1):137–142. http://dx.doi.org/10.1016/j.urology.2007.03.003.
18. Stankiewicz E, Ng M, Cuzick J, et al. The prognostic value of Ki-67 expression in penile squamous cell carcinoma. *J Clin Pathol* 2012;65(6):534–537. http://dx.doi.org/10.1136/jclinpath-2011-200638.
19. Andersson P, Kolaric A, Windahl T, Kirrander P, Söderkvist P, Karlsson MG. PIK3CA, HRAS and KRAS gene mutations in human penile cancer. *J Urol* 2008;179(5):2030–2034. http://dx.doi.org/10.1016/j.juro.2007.12.040.
20. Bienstock RJ, Barrett JC. KAI1, a prostate metastasis suppressor: prediction of solvated structure and interactions with binding partners; integrins, cadherins, and cell-surface receptor proteins. *Mol Carcinog* 2001;32(3):139–153. http://www.ncbi.nlm.nih.gov/pubmed/11746826. Accessed 16.02.15.
21. Protzel C, Kakies C, Kleist B, Poetsch M, Giebel J. Down-regulation of the metastasis suppressor protein KAI1/CD82 correlates with occurrence of metastasis, prognosis and presence of HPV DNA in human penile squamous cell carcinoma. *Virchows Arch* 2008;452(4):369–375. http://dx.doi.org/10.1007/s00428-008-0590-0.
22. Minardi D, d'Anzeo G, Lucarini G, et al. D2-40 immunoreactivity in penile squamous cell carcinoma: a marker of aggressiveness. *Hum Pathol* 2011;42(11):1596–1602. http://dx.doi.org/10.1016/j.humpath.2010.12.020.
23. Chaw SY, Majeed AA, Dalley AJ, Chan A, Stein S, Farah CS. Epithelial to mesenchymal transition (EMT) biomarkers—E-cadherin, beta-catenin, APC and Vimentin—in oral squamous cell carcinogenesis and transformation. *Oral Oncol* 2012;48(10):997–1006. http://dx.doi.org/10.1016/j.oraloncology.2012.05.011.
24. Campos RSM, Lopes A, Guimarães GC, Carvalho AL, Soares FA. E-cadherin, MMP-2, and MMP-9 as prognostic markers in penile cancer: analysis of 125 patients. *Urology* 2006;67(4):797–802. http://dx.doi.org/10.1016/j.urology.2005.10.026.
25. Han Z, He H, Bi X, et al. Expression and clinical significance of CD147 in genitourinary carcinomas. *J Surg Res* 2010;160(2):260–267. http://dx.doi.org/10.1016/j.jss.2008.11.838.
26. Protzel C, Richter M, Poetsch M, et al. The role of annexins I, II and IV in tumor development, progression and metastasis of human penile squamous cell carcinomas. *World J Urol* 2011;29(3):393–398. http://dx.doi.org/10.1007/s00345-010-0575-x.
27. Gunia S, Jain A, Koch S, et al. Periostin expression correlates with pT-stage, grading and tumour size, and independently predicts cancer-specific survival in surgically treated penile squamous cell carcinomas. *J Clin Pathol* 2013;66(4):297–301. http://dx.doi.org/10.1136/jclinpath-2012-201262.
28. Minardi D, Lucarini G, Filosa A, et al. Prognostic value of CD44 expression in penile squamous cell carcinoma: a pilot study. *Cell Oncol* 2012;35(5):377–384. http://dx.doi.org/10.1007/s13402-012-0098-0.
29. Kato H, Torigoe T. Radioimmunoassay for tumor antigen of human cervical squamous cell carcinoma. *Cancer* 1977;40(4):1621–1628. http://www.ncbi.nlm.nih.gov/pubmed/332328. Accessed 17.02.15.
30. Laniado ME, Lowdell C, Mitchell H, Christmas TJ. Squamous cell carcinoma antigen: a role in the early identification of nodal metastases in men with squamous cell carcinoma of the penis. *BJU Int* 2003;92(3):248–250. http://www.ncbi.nlm.nih.gov/pubmed/12887477. Accessed 08.02.15.
31. Touloupidis S, Zisimopoulos A, Giannakopoulos S, Papatsoris AG, Kalaitzis C, Thanos A. Clinical usage of the squamous cell carcinoma antigen in patients with penile cancer. *Int J Urol* 2007;14(2):174–176. http://dx.doi.org/10.1111/j.1442-2042.2007.01694.x.
32. Rosevear HM, Williams H, Collins M, Lightfoot AJ, Coleman T, Brown JA. Utility of ^{18}F-FDG PET/CT in identifying penile squamous cell carcinoma metastatic lymph nodes. *Urol Oncol* 2012;30(5):723–726. http://dx.doi.org/10.1016/j.urolonc.2010.09.015.
33. Muñoz N. Human papillomavirus and cancer: the epidemiological evidence. *J Clin Virol* 2000;19(1-2):1–5. http://www.ncbi.nlm.nih.gov/pubmed/11091143. Accessed 30.09.13.
34. Ghittoni R, Accardi R, Hasan U, Gheit T, Sylla B, Tommasino M. The biological properties of E6 and E7 oncoproteins from human papillomaviruses. *Virus Genes* 2010;40(1):1–13. http://dx.doi.org/10.1007/s11262-009-0412-8.
35. Rubin MA, Kleter B, Zhou M, et al. Detection and typing of human papillomavirus DNA in penile carcinoma: evidence for multiple independent pathways of penile carcinogenesis. *Am J Pathol* 2001;159(4):1211–1218. http://dx.doi.org/10.1016/S0002-9440(10)62506-0.
36. Maden C, Sherman KJ, Beckmann AM, et al. History of circumcision, medical conditions, and sexual activity and risk of penile cancer. *J Natl Cancer Inst* 1993;85(1):19–24. http://www.ncbi.nlm.nih.gov/pubmed/8380060. Accessed 25.02.15.
37. Castellsagué X, Bosch FX, Muñoz N, et al. Male circumcision, penile human papillomavirus infection, and cervical cancer in female partners. *N Engl J Med* 2002;346(15):1105–1112. http://dx.doi.org/10.1056/NEJMoa011688.
38. Lont AP, Kroon BK, Horenblas S, et al. Presence of high-risk human papillomavirus DNA in penile carcinoma predicts favorable outcome in survival. *Int J Cancer* 2006;119(5):1078–1081. http://dx.doi.org/10.1002/ijc.21961.
39. *Cancer Research UK Biomarker Roadmap*. http://www.cancerresearchuk.org/prod_consump/groups/cr_common/@fre/@fun/documents/generalcontent/cr_027223.pdf. Accessed 14.02.15.
40. Feber A, Arya M, de Winter P, et al. Epigenetics markers of metastasis and HPV induced tumourigenesis in penile cancer. *Clin Cancer Res* 2014. http://dx.doi.org/10.1158/1078-0432.CCR-14-1656.