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Title: Over-expression of DNMT3A predicts the risk of recurrent vulvar squamous cell carcinomas

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ABSTRACT

Objective: Cancer initiation and progression has been linked to aberrant expression of the DNA methyltransferases (DNMT), the enzymes which establish and maintain DNA methylation patterns throughout the genome. In this study, we investigated if DNMT expression in vulvar squamous cell carcinomas (VSCC) was related to clinical outcome.

Methods: DNMT1, DNMT3A and DNMT3B expression was measured in a subset of cases drawn from a cohort of consecutive women treated for primary VSCC at the Pan Birmingham Gynaecological Cancer Centre between 2001 and 2008. Univariable and multivariable competing risk modelling was performed to identify whether DNMT expression was associated with local disease recurrence or disease morbidity.

Results: Over-expression of DNMT3A in the invasive component of the tumour was seen in 44% of tumours and was associated with an increased risk of local vulval recurrence (LVR) (HR = 4.51, p = 0.012). This risk was found to increase further after adjustment for disease stage (HR = 6.00, p = 0.003) and groin node metastasis (HR = 4.81, p = 0.008). Over-expression of DNMT3B was associated with an increased risk of LVR (HR = 5.69 p = 0.03), however this ceased to be significant after adjustment for groin node metastasis. In a subset analysis, over-expression of DNMT3A was found to be significantly more common in VSCCs that stained negative for CDKN2A.

Conclusions: These observations are consistent with the possibility that epigenetic changes contribute to vulval neoplasia and DNMT3A over-expression may be useful in predicting local disease recurrence.

Introduction

Vulvar squamous cell carcinoma (VSCC) is relatively rare, accounting for approximately 5% of all gynecological malignancies and 95% of all vulvar malignancies [1]. However the incidence of VSCC has recently been rising, leading to increased clinical and scientific interest to improve therapeutic options [2-4]. Over the last two decades evidence has accumulated to show there are two different aetio-pathogenic pathways of the precursor lesions, termed vulvar intra-epithelial neoplasia (VIN) [5]. The first, usual type vulvar intraepithelial neoplasia (uVIN) is related to human papillomavirus (HPV) infection, predominantly type 16. The second pathway which is independent of HPV infection is referred to as differentiated VIN (dVIN) [6], and is often associated with Lichen Sclerosis (LS), a non-neoplastic, chronic inflammatory dermatosis with distinctive histology [7,8]. Although there is evidence to suggest that HPV-derived tumours confer a better survival over their HPV-independent counterpart in squamous cell cancer of the head and neck, such an advantage in survival is yet to be established in VSCC [9]. The rate of local vulval recurrence (LVR) has not changed over the last 3 decades and remained at around 25% despite the introduction of chemo-radiotherapy as an adjunct to surgery [10-12]. Our recent cohort study suggests that women with VSCC arising in the background of LS were more likely to develop LVR even when the primary tumours were completely excised with adequate excision margins [13].

DNA methylation is a covalent modification of DNA tightly regulated by a group of three enzymes known as the DNA methyltransferases (DNMT). In normal cells DNMT1 is primarily responsible for maintaining methylation; however its importance in maintaining cancer stem cells and driving tumorigenesis is also well established [14-16]. DNTM3A and DNMT3B are active *de novo* methyltransferases, although expressed at lower levels in adult somatic cells, they have been shown to be overexpressed and associated with adverse prognosis in several tumours including those of epithelial origin [17-19]. A recent study has shown that tumour suppressor gene (TSG) methylation in VSCC is associated with a poor disease prognosis however whether DNMT expression could be useful in determining disease prognosis remains unclear [20].

In this study, we compare for the first time, the expression of DNMT1, DNMT3A and DNMT3B in the invasive component and in the normal adjacent epithelium of 66, 66 and 62 VSCC cases respectively, and associated their expression with clinical data collected from patient's medical records and CDKN2A status.

Materials and methods

Vulval tumour study population:

DNMT expression was examined in a subset of cases drawn from a cohort of 201 consecutive patients first diagnosed with VSCC between 2000 and 2008. All patients were actively followed up for at least 5 years with cause of death assigned using information from multiple sources which included hospital and hospice notes, general practitioner summaries, cancer registry records and information supplied by the patient's family. Information on the following variables from case notes was documented: age, smoking behaviour, multifocal disease, tumour differentiation, lymphovascular space involvement, stage, groin node involvement and the presence of concomitant non-neoplastic epithelial disorders. Information on local, regional and distant recurrences was also recorded during the follow-up period. Tissue was obtained with written consent and the use of the biopsy material for research purposes had been approved by the Birmingham, East, North and Solihull Research Ethics Committee (Reference number 11/WM/0070).

Preparation of study material:

Paraffin embedded blocks were sectioned every 4µm and one section stained with haematoxylin and eosin; sequential sections were used for routine immunohistochemical staining with the last of these sections used for HPV DNA testing as described previously [13]. To avoid cross contamination, the microtome blades were changed between cutting of each block. Tumour enriched tissue was macro dissected and DNA was extracted using AllPrep DNA/RNA FFPE (formalin fixed paraffin embedded) kit, according to manufacturer's instructions (Qiagen) and stored at -20^{o}C.

Immunohistochemical analysis:

DNMT immunohistochemistry

Immunohistochemistry to examine the expression of DNMT1, DNMT3A and DNMT3B was performed on 66, 66 and 62 VSCC cases respectively. We included only those cases which had tumour and adjacent normal epithelium, and which performed similarly in two independent experiments to control for batch variation; thus accounting for the number of cases used in this study. Antigen expression was assessed using methods we have previously described [21]. Primary antibodies to DNMT1 (ab19905 – ABCAM), DNMT3A (HPA026588-SIGMA) and DNMT3B (HPA001595-SIGMA) were used at optimal working dilutions of 1:1200, 1:200, and 1:800 respectively. FFPE tissues from tonsil and skin were used as controls.

Two pathologists (RG, MP), independently reviewed the IHC stained slides. Distinctive brown DAB nuclear staining was considered as positive for DNMT1, DNMT3A and DNMT3B protein expression. All invasive vulvar cancer tissue sections were initially scanned at x4 objective to select the most tumour dense area. Then one field at x10 objective was selected and the total number of DNMT expressing tumour cells was assessed. When assessing keratinising sub-types of VSCC, it was noted that the lack of nuclei in the keratin layer result in an apparent lack of staining. In such instances, staining of nucleated layers was assessed.

CDKN2A immunohistochemistry

43 tumours which were stained for DNMT expression were also tested for CDKN2A expression. A primary antibody to CDKN2A (clone E6H4 – CINtec) was used at an optimal working dilution of 1:25. Expression was considered positive when there was diffuse block staining of a segment of epithelium. Patchy staining, even of strong intensity, or staining of varied intensity was considered negative [22].

H scoring

The H-score scoring system was used to quantify the immunohistochemical staining. The score is based on the proportion of target nuclei stained and the intensity of the stain [23]. The formula used is: 3 x percentage of strongly stained nuclei (colour = brownish black) + 2 x percentage of moderately

stained nuclei (colour = brown) + 1 x percentage of weakly stained nuclei (colour = light brown) + 0 x percentage of absent staining nuclei. Thus the H-score generated a range from 0 to 300, where 300 is equivalent to 100% of the cells of interest exhibiting strong staining. DNMT were considered over-expressed when the H score of the tumour was greater than that of the normal adjacent epithelium in both replicates.

Statistical analysis:

Survival was summarised in each DNMT expression subgroup using the methods of Kaplan and Meier. Univariate Cox models were used to quantify the association between cohort characteristics and the risk of disease specific survival and time to local vulval recurrence, specifically the impact of overexpression of DNMT markers. Multivariable Cox modelling was performed to adjust the estimates of risk associated DNMT expression for known confounders. Given the limited size of each cohort there was scope to adjust for just one confounder other than the DNMT descriptor in each multivariable model. The patient characteristics were explored within each sub-cohort by DNMT expression through comparison of proportions via a chi-squared test, and comparison medians using a Wilcoxon rank-sum test. P values <0.05 were deemed statistically significant. Analyses were performed in Stata V12.1

Pyrosequencing:

Genomic DNA (500 ng) was bisulfite converted using EZ DNA methylation kit (Zymo Research). Pyrosequencing primers used for each of the promoter regions are shown in Supplemental table 1

The PCR was performed in a total volume of 50 µl using 25 µl HotStart Taq master mix (Thermo Scientific) with 10pmol of primers and 10 µl of bisulfite-modified DNA. The pyrosequencing reactions were performed on a Pyromark ID system (Qiagen) and analysed using Pyro Q-CpG software (Qiagen).

Results

Expression of DNMT1, DNMT3A and DNMT3B in VSCC

DNMT1, DNMT3A and DNMT3B expression in the invasive component was compared to that in the normal adjacent epithelium. Replicate cases which were not reproducible were removed from our analysis. Therefore DNMT1, DNMT3A and DNMT3B expression in relation to prognosis was analysed in 66, 55 and 45 VSCC cases respectively. DNMT1 was over-expressed in 83% (55/66) of tumours, DNMT3A was over-expressed in 44% (24/55) and DNMT3B was over-expressed in 42% (18/45) of tumours contributing to this analysis. For every case with DNMT over-expression, all tumour cells stained positive in the invasive component. Representative examples of VSCC showing over-expression of DNMT3A, DNMT3B and DNMT1 compared to the normal adjacent epithelium (NAE) is shown in Figure 1.

Risk of local vulval recurrence is associated with DNMT3A over-expression

We set out to determine whether DNMT1, DNMT3A or DNMT3B over-expression is associated with the risk of LVR.

DNMT3A: 13 of the 55 women in the DNMT3A cohort had a LVR. LVR free survival at 1, 3 and 5 years was found to be 75.4%, 56.3% and 47.5%, respectively, when DNMT3A was over-expressed, and 96.4%, 92.6% and 83.3% when it was not (Figure 2A). In a univariable analysis, DNMT3A over-expression was found to be associated with a 4.5 fold increased risk of LVR (HR=4.51, p= 0.012). Risk of LVR was also increased in patients with groin node metastasis (HR=4.72, p=0.005) (Table 1). In a multivariable analysis, DNMT3A over-expression continued to be associated with increased risk of developing a LVR after adjustment for disease stage (HR=6.00, p=0.003), groin node metastasis (HR=4.81, p=0.008), and the presence of LS adjacent to the primary tumour (HR=4.08, p=0.019).

DNMT3B: 9 of the 45 women in the DNMT3B cohort had a LVR. LVR free survival at 1, 3 and 5 years was found to be 88.5%, 63.0% and 56.8%, respectively, when DNMT3B was over-expressed,

and 100%, 94.1% and 77.4% when it was not (Figure 2A). Like DNMT3A, univariable analysis indicated that DNMT3B over-expression was associated with a significantly increased risk of LVR (HR=5.69, p=0.03) (Table 1). Risk of LVR was also increased in women with groin node metastasis (HR=4.07, p=0.04) and in those with tumours arising in a background of LS (HR=11.25, p=0.023) (Table 1). In a multivariable analysis, DNMT3B over-expression continued to be associated with increased risk of developing a LVR after adjustment for disease stage (HR=4.80, p=0.029). Unlike DNMT3A, the excess risk associated with DNMT3B over-expression was attenuated and ceased to be significant after adjustment for groin node metastasis (HR=4.18, p=0.088) and the presence of LS adjacent to the primary tumour (HR=2.99, p=0.19)

DNMT1: 14 of the 66 women in the DNMT1 cohort had a LVR. LVR free survival at 1, 3 and 5 years was found to be 91.2%, 81.3% and 75.0%, respectively, when DNMT1 was over-expressed and 90.0%, 90.0% and 64.3% when it was not (Figure 2A). Unlike DNMT3A and DNMT3B, univariable analyses resulted in no evidence that DNMT1 over-expression increases risk of LVR (HR=0.77, p=0.691) (Table 1). Risk of LVR was substantially increased in women with groin node metastasis (HR=4.84, p=0.006) and in those with tumours arising in a background of LS (HR=4.41, p=0.012) (Table 1). The risk of LVR associated with DNMT1 over-expression remained insignificant after adjustment.

DNMT3A, DNMT3B or DNMT1 expression does not influence disease specific mortality

We next set out to determine whether DNMT1, DNMT3A or DNMT3B expression influences disease mortality. However we found no significant difference in disease specific survival at 1, 3 and 5 years for each of the DNMT (Figure 2B). In both univariable and multivariable analysis DNMT1, DNMT3A and DNMT3B over-expression was not associated with increased mortality (Table 2).

Over-expression of DNMT3A is associated with CDKN2A expression

43 tumours from the VSCC cohort were tested for CDKN2A expression, which is indicative of HPV induced dysplasia [22]. Of the 39 tumours for which CDKN2A result was available, 15 (38.5%)

showed positive block staining which is characteristic of a transforming HPV infection and 24 were negative for CDKN2A expression (Figure 3A and B). When we compared CDKN2A and DNMT expression, we found that in the 24 tumours negative for CDKN2A that over-expression of DNMT3A was significantly more common (33.3% vs. 0%, $_{\text{difference in proportions}}$ 33.3%, 95% CI 2.34-56.25). Representative examples showing CDKN2A expression in the absence of DNMT3A and vice versa are shown in Figure 3. The expression of DNMT1 and DNMT3B did not vary significantly with CDKN2A expression. We next examined the methylation status of the CDKN2A promoter using pyrosequencing and found that CDKN2A negative tumours were significantly more methylated than those tumours which were CDKN2A positive (T-test 0.0014) (Figure 4). Although the observations in our results point to a relationship between DNMT3A over-expression and the absence of CDKN2A expression, and a relationship between the absence of CDKN2A expression and hypermethylation of the CDKN2A promoter, we were not able to directly link the over-expression of DNMT3A to hypermethylation of the CDKN2A promoter. Although we show an association between DNMT3A over expression and CDKN2A expression (indicative of HPV induced dysplasia), there was no association between DNMT3A over-expression and HPV status when this was assigned using PCR based assays.

Discussion

Approximately one in four women with VSCC will have a LVR within five years after primary surgery [13]. Known risk factors for LVR are the clearance margin of the tumour, the depth of invasion by tumour cells and, as we reported recently, the presence of LS adjacent to the tumour [24, 13]. However, more sophisticated predictors such as biomarkers capable of distinguishing cases likely to recur, are of clinical importance.

Maintenance of DNA methylation by the DNMT is critical during development and in transcriptional regulation; however aberrant expression of the DNMT has been reported for a number of human cancers, including those of epithelial origin [16, 17, 19]. To date, expression of the DNMT in VSCC has not been examined, therefore, we evaluated the expression of these enzymes and correlated our

results with CDKN2A staining, clinicopathologic parameters and survival data to determine the prognostic significance of DNMT expression in vulvar cancer.

Our data showed that DNMT3A over-expression but not DNMT1 or DNMT3B was associated with a significantly increased risk of LVR. This increased risk persisted after adjustment for groin node metastasis suggesting that over-expression is an important predictor of VSCC recurrence. It has also been shown for gastric carcinoma that DNMT3A over-expression while not DNMT3B or DNMT1 is associated with poor disease survival [25]. For both epithelial tumours over-expression of DNMT3A significantly contributes to disease recurrence or progression which may be associated with the disruption of similar molecular pathways, such as epithelial-to-mesenchymal transition (EMT). During EMT cells undergo a developmental switch from a polarized, epithelial phenotype to a highly motile fibroblastoid or mesenchymal phenotype, a central process during cancer progression [26]. It is recognised that VSCC with prominent fibromyxoid stroma have a poorer outcome. This stromal response is phenotypic of epithelial mesenchymal transition [27]. Vulval cancers like other epithelial malignancies, express a set of proteins which characterize strongly the transition from normal epithelium to invasive VSCC [28]. The importance of DNMT3A in the regulation of these proteins during EMT has recently been highlighted by Tan et al, where they show that a non-histone chromatin protein, HMGA2 remodels chromatin to favour binding of DNMT3A to the *CDH1* promoter, thus achieving sustained silencing of E-cadherin expression and promoting tumour cell invasion [29].

Our results also showed that DNMT3A overexpression was inversely related to the expression of CDKN2A in these carcinomas. Expression of CDKN2A is related to the regulation of Rb by the viral oncogene, E7. Following HPV integration into the host genome, E7 expression increases as does CDKN2A expression due to its accumulation in the cell [30]. These results may tie in with a recent finding published by Rodriguez et al, which suggests that HPV positive vulvar tumours may not progress through EMT while HPV negative tumours do, again suggesting a role for DNMT3A in the EMT process [28]. The presence of HPV when detected using PCR based methods did not reveal an

association with DNMT3A over expression, however this may not be surprising given that it may only be transcriptionally active HPV that is related to disease progression [22].

Our results showed that over expression of DNMT3A and DNMT3B was associated with an LVR but not survival. Identifying factors which are associated with local recurrence but not survival is not unusual; in fact, these results are consistent with findings in our cohort study which shows that mortality/survival is not influenced by LVR [13]. We also found in this study that women who developed LVR also went on to develop at least one further episode of LVR within 2.3 years [13]. These findings might also suggest that DNMT3A is over expressed in a subset of less aggressive tumours, a type associated with recurrence but not death. However given this is the first time we have established this association, further work on the molecular biology of these VSCC tumours would be necessary to fully understand this.

In summary, we have demonstrated that molecular profiling of the tumour and its adjacent epithelium may be used to predict treatment outcomes in patients with VSCC. We have shown, for the first time that DNMT3A over-expression is associated with tumours that do not contain transcriptionally active HPV and have an increased risk of LVR. Our study suggests that DNMT3A may be used as a surrogate marker for HPV-negative tumours and serve as a biomarker to identify those patients who are at risk of developing LVR. Furthermore, given that a family of propiophenone derivatives have recently been shown to be specific DNMT3A inhibitors in cancer cells, raises the interesting possibility of testing these novel agents for their use in the prevention of some vulvar recurrences [31]. This of course requires further evaluation including clinical studies.

Conflict of interest

The authors declare no conflict of interest.

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Figure and Table Legends

Figure 1: Examples of VSCC with over-expression of DNMT1, DNMT3A or DNMT3B. The H score of the invasive areas of the tumour was compared with that of the non-neoplastic squamous epithelium lying adjacent to the invasive lesion (NAE).

Figure 2A: Univariate analysis showing a statistically significant increased risk of local vulval recurrence when there is over-expression of DNMT3A and DNMT3B but not DNMT1. 2B: Univariate analysis showing a statistically insignificant risk of disease specific survival (i.e. VSCC related death) when there is over-expression of DNMT3A, DNMT3B and DNMT1.

Figure 3: Low level DNMT3A expression in tumours with CDKN2A overexpression. A representative example of a tumour in which both the immature keratinising and keratinising components of the tumour show strong CDKN2A expression but low level DNMT3A expression.

Figure 4: Graph showing the average methylation of the P16 promoter in 43 VSCC tumours.

Table 1: Univariable analysis showing the risk of Local Vulval Recurrence in relation to DNMT expression and patient and disease related prognostic factors.

Table 2: Univariable analysis showing the risk of death from VSCC in relation to DNMT over-expression and patient and disease related prognostic factors.

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Covariate		DNMT 1 (N=66)		DNMT3A (N=55)		DNMT3B (N=45)	
		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	P-value
DNMT over-expression	Not over-expressed	1 (reference)		1 (reference)		1 (reference)	
	Over-expressed	0.77 (0.21, 2.77)	0.691	**4.51 (1.40, 14.49)**	**0.012**	**5.69 (1.17, 27.57)**	**0.031**
Age	≤70	1 (reference)		1 (reference)		1 (reference)	
	>70	1.16 (0.40, 3.36)	0.781	0.73 (0.25, 2.11)	0.563	0.55 (0.14, 2.21)	0.400
Smoking status	Smoker/Ex-Smoker	1 (reference)		1 (reference)		1 (reference)	
	No	7.42 (0.96, 57.32)	0.055	3.76 (0.83, 17.06)	0.086	3.28 (0.40, 26.81)	0.269
	Not mentioned	1.88 (0.12, 30.10)	0.657	0.57 (0.05, 6.25)	0.642	1.06 (0.07, 16.99)	0.968
Stage if disease	1/2	1 (reference)		1 (reference)		1 (reference)	
	3/4	2.8 (0.95, 8.25)	0.062	3.34 (1.14, 9.77)	0.028	1.69 (0.15, 33.35)	0.437
Tumour size	≤4cm	1 (reference)		1 (reference)		1 (reference)	
	> 4cm	1.16 (0.39, 3.46)	0.789	1.07 (0.36, 3.18)	0.909	0.98 (0.45, 6.35)	0.990
Multifocal disease	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	1.37 (0.43, 4.39)	0.593	1.71 (0.54, 5.47)	0.363	1.04 (0.22, 5.04)	0.96
Groin node involvement	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	**4.84 (1.56, 15.07)**	**0.006**	**4.72 (1.60, 13.93)**	**0.005**	**4.07 (1.06, 15.59)**	**0.04**
Lymphovascular space involvement	No	1 (reference)		1 (reference)		1 (reference)	
	Unavailable	1.78 (0.48, 6.60)	0.388	2.20 (0.55, 8.79)	0.266	0.00 (0.00, .)	1
	Yes	0.37 (0.08, 1.73)	0.209	1.20 (0.37, 3.95)	0.76	0.63 (0.16, 2.53)	0.514
Excision margin status	Optimum	1 (reference)		1 (reference)		1 (reference)	
	Incomplete	0.00 (0.00, .)	1	0.00 (0.00, .)	1	0.00 (0.00, .)	1
	Sub-optimum	0.85 (0.26, 2.76)	0.786	0.72 (0.22, 2.33)	0.58	0.82 (0.20, 3.30)	0.781
	Unavailable	4.05 (0.49, 33.68)	0.196	3.45 (0.41, 28.78)	0.253	1.00 (., .)	.
Tumour differentiation	Well	1 (reference)		1 (reference)		1 (reference)	
	Moderate	1.56 (0.41, 5.83)	0.512	1.24 (0.33, 4.61)	0.751	1.09 (0.22, 5.44)	0.917
	Poorly	0.93 (0.22, 3.91)	0.924	0.87 (0.23, 3.26)	0.84	0.49 (0.08, 2.96)	0.438
	Not graded	1.93 (0.37, 9.98)	0.431	0.00 (0.00, .)	1	0.76 (0.08, 7.40)	0.816
LS+/-VIN	No LS	1 (reference)		1 (reference)		1 (reference)	
	LS, +/- Vin	**4.41 (1.38, 14.07)**	**0.012**	2.47 (0.83, 7.36)	0.106	**11.25 (1.40, 90.14)**	**0.023**

Table 1

Covariate		DNMT1 (N=66)		DNMT3A (N=55)		DNMT3B (N=45)	
		HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
DNMT over-expression	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	0.85 (0.28, 2.56)	0.772	2.02 (0.66, 6.21)	0.219	1.48 (0.45, 4.86)	0.517
Age	≤70	1 (reference)		1 (reference)		1 (reference)	
	>70	1.34 (0.53, 3.42)	0.535	2.35(0.71, 7.77)	0.162	0.82 (0.25, 2.69)	0.740
Smoking status	Smoker/Ex-Smoker	1 (reference)		1 (reference)		1 (reference)	
	Never smoker	1.24 (0.39, 3.94)	0.721	1.08 (0.27, 4.33)	0.917	0.57 (0.13, 2.57)	0.466
	Not mentioned	2.26 (0.60, 8.57)	0.23	1.91 (0.42, 8.77)	0.403	1.25 (0.28, 5.63)	0.775
Stage of disease	1/2	1 (reference)		1 (reference)		1 (reference)	
	3/4	9.95 (3.26, 30.43)	0.000	6.27 (1.71, 22.98)	0.006	6.94 (1.49, 32.3)	0.014
Tumour size	≤4cm	1 (reference)		1 (reference)		1 (reference)	
	>4cm	3.63 (1.28, 10.26)	0.015	4.86 (1.25, 18.87)	0.022	1.92 (0.54, 6.83)	0.312
Multifocal disease	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	0.69 (0.20, 2.37)	0.551	0.80 (0.18, 3.61)	0.77	0.89 (0.19, 4.15)	0.884
Groin node involvement	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	9.68 (3.51, 26.66)	<0.01	6.16 (1.87, 20.31)	0.003	6.50 (1.66, 25.5)	0.007
Lymphovascular space involvement	No	1 (reference)		1 (reference)		1 (reference)	
	Yes	1.30 (0.48, 3.49)	0.607	2.07 (0.58, 7.33)	0.261	1.79 (0.52, 6.17)	0.356
	Unavailable	1.66 (0.45, 6.17)	0.446	2.61 (0.57, 11.87)	0.214	0.00 (0.00, .)	1
Excision margin status	Optimum	1 (reference)		1 (reference)		1 (reference)	
	Incomplete	3.92 (1.00, 15.29)	0.049	7.82 (0.83, 73.32)	0.072	3.76 (0.41, 34.8)	0.243
	Sub-optimum	1.84 (0.60, 5.64)	0.289	2.79 (0.77, 10.07)	0.118	1.52 (0.38, 6.13)	0.554
	Unavailable	7.96 (1.98, 31.95)	0.003	10.73 (1.82, 63.33)	0.009	1.3e+18 (., .)	.
Tumour differentiation	Well	1 (reference)		1 (reference)		1 (reference)	
	Moderate	1.89 (0.53, 6.72)	0.326	1.27 (0.34, 4.80)	0.724	1.29 (0.29, 5.85)	0.738
	Poorly	2.90 (0.95, 8.89)	0.062	0.85 (0.21, 3.45)	0.818	1.13 (0.25, 5.07)	0.87
	Not graded	0.00 (0.00, .)	1	0.00 (0.00, .)	1	0.00 (0.00, .)	1
LS+/-VIN	No LS	1 (reference)		1 (reference)		1 (reference)	
	LS, +/- Vin	1.41 (0.57, 3.47)	0.458	0.92 (0.30, 2.82)	0.887	3.22 (0.85, 12.1)	0.084

Table 2