Original Articles

Intratumoral oxygenation of invasive squamous cell carcimoma of the vulva is not correlated with regional lymph node metastasis

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Summary

Introduction: Tumour hypoxia has been found to be associated with tumour aggressiveness. Our primary aim was to explore the relationship between pretreatment tumour oxygenation in primary vulvar carcinoma and nodal status. Our secondary objective was to assess if there was a relationship between the clinical and biological variables.

Methods: 20 women with ISCC of the vulva were assessed with pretreatment primary tumour oxygenation with an Eppendorf pO_2 probe. Patients underwent standard surgical management. Pathological assessment of the primary and nodal tissues was then performed. Primary tumour specimens were also stained for microvessel density and carbonic anhydrase IX. The relationship between smoking, preoperative Hgb, tumour CAIX expression, MVD, and Eppendorf pO_2 measurements vs nodal metastasis and between these clinical and biological variables was assessed.

Results: Seven patients had positive lymph nodes, 13 had negative nodes. While neither current smoking status, tumour size, tumour oxygen measurements, MVD and CAIX expression correlated with metastatic nodal disease, a low preoperative Hgb correlated with pathological nodal status ($p < 0.027$).

Conclusions: Although this analysis failed to demonstrate a strong correlation between various measures of tumour oxygenation with nodal metastasis, it may be due to the small number of patients. Only preoperative anaemia is correlated with nodal metastasis in early ISCC of the vulva.

Key words: Vulvar cancer; Tumour hypoxia; Nodal metastasis.

Introduction

Invasive squamous cell carcinoma (ISCC) of the vulva represents approximately 3-4% of malignancies of the female genital tract. Historically ISCC of the vulva is a disease affecting postmenopausal women, however, recent studies suggest a bimodal distribution of the disease, with increasing numbers of women in their 30's being diagnosed [1]. Management of ISCC is by vulvectomy and regional lymphadenectomy. Morbidity associated with this procedure includes wound dehiscence (15-50%) and lower extremity lymphedema (chronic in 30%) [2, 3]. Lymphadenectomy is useful in terms of staging the disease but it is not considered therapeutic.

Little information, aside from primary tumour size, histologic grade and loco-regional anatomical spread of disease [4-6] predicts nodal metastasis in vulvar cancer. Pretreatment intra-tumoral hypoxia as measured with the Eppendorf pO_2 histograph has been found to be prognostic in cancers of the head and neck, cervix, lung and soft tissue sarcomas and predicts both local control as well as the likelihood of distant metastasis [7-11]. In addition intrinsic markers of tumour hypoxia have also been shown to be of prognostic value [12]. To date, there are no published reports assessing tumour oxygenation in vulvar cancer using the oxygen electrode. With the use of this tool, along with a variety of indirect markers of oxygenation, we attempted to identify markers that could preoperatively predict nodal metastasis in vulvar cancer. The purpose of this study was to assess the oxygen tension measured with the Eppendorf pO_2 histograph probe in primary vulvar tumors prior to therapy and to correlate this with node status. If a strong correlation is found, the need for routine lymphadenectomy in all patients with ISCC could be re-visited.

To assess the hypothesis that tumour hypoxia relates to tumour aggressiveness we assessed tumour oxygenation using other markers to include MVD and CA IX, both of which have been shown to be prognostic in solid tumours [12-18]. Also both of these have been shown to be prognostic indictors in squamous cell tumours [19-21].

Materials and Methods

Study Group

Between 1998-2001, 20 women with ISCC of the vulva entered into a protocol assessing pretreatment primary tumour oxygenation with an Eppendorf pO_2 probe. Eligibility criteria included pathological confirmation of ISCC, visible and palpable lesions ≥ 1.5 cm, no prior therapy for this malignancy and

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planned primary therapy of surgical resection with regional lymphadenectomy. Ineligibility criteria included inability to provide informed consent, inability to tolerate a non-anaesthetized clinical examination of the vulva or presence of a significant bleeding disorder. Patients were assessed at the initial consultation to determine suitability for entry into the study and, if eligible, informed consent was obtained. A retrospective chart review was performed for each patient. Demographic as well as co-morbid disease information was collected.

Oxygen tension measurements

Patients underwent oxygen tension measurements in the outpatient department of the clinic. No local, regional or general anaesthesia was employed. The Eppendorf pO_2 probe was inserted into a clinically representative region of the tumour. The technical details are provided elsewhere [22]. Oxygen tension measurements were obtained along a number of linear tracks within the tumour. The track length, number and location were determined at the discretion of the investigator. PO_2 values corresponding to the median, % of values ≤ 2.5 and % of values ≤ 5 mm Hgb were used for analysis.

Pathological assessment

Following surgical resection and regional lymphadenectomy, the surgical specimen was processed according to the standard protocol at the host institution. Pathological confirmation of the invasive primary and the absence or presence of nodal spread was established. Immunohistochemical stains were then performed on surgical tumour specimens to assess microvessel density (MVD) and carbonic anhydrase IX (CAIX) expression.

Determination of microvessel density

For each case, one representative block of formalin-fixed, paraffin-embedded vulvar tissue containing invasive squamous cell carcinoma was selected for microvessel staining and counting. Four micron thick sections were cut and transferred to silastin-coated slides. The endothelial cells of blood vessels were highlighted with a polyclonal CD 34 antibody (Dako, Glostrup, Denmark). Briefly, sections were deparaffinized and rehydrated in graded alcohol solutions. Heat induced antigen retrieval was achieved in a pressure cooker that was heated in a microwave for 35 minutes and then cooled for 20 minutes. The CD 34 antibody was applied at a 1:50 dilution, and staining was performed on a Ventana ES immunostainer (Ventana Medical Systems, AZ). A streptavidin-based detection system and AEC chromagen were used. Sections were counter-stained with hematoxylin.

Microvessel density was assessed without knowledge of patient nodal status. Tumour sections were initially scanned at low power (40x) to determine the areas of highest neovascularization. Within these areas, individual microvessels were counted in three separate high power (200x) fields. Only microvessels within the confines of invasive tumour were counted. Areas of haemorrhage, necrosis and ulceration were avoided.

For each case, the mean microvessel count for the three fields assessed was calculated. A microvessel was defined as any single endothelial cell or group of endothelial cells stained, with or without a discernible vessel lumen, that was clearly separate from adjacent microvessels.

Determination of CAIX expression

Immunohistochemical staining for CAIX was performed on 5-mm serial sections on coated slides from paraffin-embedded blocks. Paraffin was first removed from all slides by means of standard techniques; slides were then placed in 0.5% hydrogen peroxide for 15 minutes to saturate endogenous peroxidases. Incubation with 10% normal human serum in TBS for 15 minutes was then performed to block nonspecific uptake of the antibody. The murine monoclonal antibody at a dilution of 1:50 in TBS with 5% normal human serum for 30 minutes was used. CAIX immunostaining was assessed as follows: low - focal weak immunostaining, medium - staining of 5-50% of tumour cells, high - greater than 50% of tumour cells positive. No cases were completely negative for CAIX expression.

The Spearman's rank and Mann-Whitney U tests were used in the statistical analysis.

This study was approved by the UBC ethics committee.

Results

Twenty women were included in this report; seven (35%) were found to have metastatic nodal spread of ISCC of the vulva. The median age of the total study group was 69 years. The node negative vs node postive group did not differ in terms of a history of cardiovascular disease, current smoking status, age at diagnosis nor with respect to the presence of lichen sclerous (28 vs 30%).

Specific details about each subject's tumour pathology is provided in Table 2. The relationship between patient and primary tumour variables and nodal status was assessed with the Mann-Whitney U test. Tumour size, as clinically measured preoperatively by its maximum dimension, did not correlate with nodal status, $p = 0.699$.

Table 1. — *Patient variables.*

	All patients	Node positive	Node negative
N	20	7	13
Median age (yrs)	69	75	68
History of cardiovascular disease	10	3	7
Median preop Hgb (g/l)	134	120	139
Current smoker	9	3	6
Lichen sclerosis	6	2	4

Table 2. — *Tumour variables.*

Patient no.	Node dissection	Pathological node status	Maximum tumour dimension	FIGO Surgical Stage
1	Bilateral	+	15 cm	IVb
2	Bilateral	–	5 cm	II
3	Bilateral	+	3 cm	IVa
4	Bilateral	+	2.5 cm	IVb
5	Bilateral	+	4 cm	IVb
6	Bilateral	+	2 cm	IVb
7	Bilateral	–	3 cm	II
8	Bilateral	–	5 cm	II
9	Bilateral	–	3.5 cm	II
10	Left	–	4 cm	II
11	Bilateral	+	4 cm	IVa
12	Bilateral	–	3 cm	II
13	Bilateral	–	1.8 cm	IA
14	Right	–	4 cm	II
15	Right	+	4.9 cm	III
16	Bilateral	–	3 cm	II
17	Bilateral	–	1 cm	Ia
18	Bilateral	–	4.5 cm	II
19	Bilateral	–	3.5 cm	II
20	Left	–	2.8 cm	II

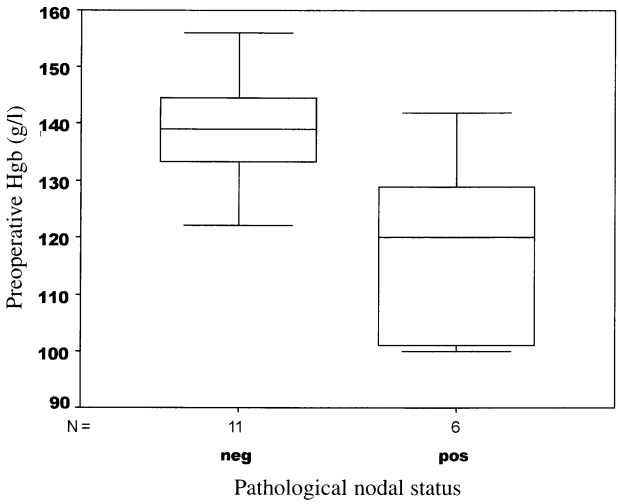

Figure 1. — Boxplot of preoperative Hgb vs nodal status.

CAIX staining was performed on 14 samples, six node positive and eight node negative. Expression was assessed as high, medium and low; no significant correlation with nodal metastasis was identified. Microvessel density was compared between node-positive and node-negative groups with a total of 17 samples available for staining (n = 6, n = 11, respectively). Again, no significant difference was observed between these groups ($p < 0.791$). Eppendorf measurements were obtained in all 20 patients. No difference between node negative and node positive subjects was found for the median pO_2 values, % values ≤ 5 mmHg or ≤ 2.5 mmHg.

Preoperative haemoglobin values were available for 17 of the 20 patients. The Hgb values in those patients found to have nodal spread were significantly lower than those in whom no nodal disease was found: 118 vs 136 g/l ($p < 0.027$) (Figure 1).

In exploring the relationship between patient-related factors and tumour factors using Spearman's correlation test, a weak correlation was found between the MVD and percentage of Eppendorf values ≤ 5mmHgb ($p = 0.031$, $r^2 = .28$).

Discussion

A number of studies have investigated prognostic factors in ISCC of the vulva; univariate analysis has shown that obesity, smoking, diabetes and hypertension are associated with decreased survival. In terms of predicting nodal spread however, only tumour size, grade and depth of invasion have been identified as reliable indicators of metastasis [4, 23-27]. The purpose of this study was to assess if primary tumour oxygenation could predict nodal spread of ISCC of the vulva. In doing so, the need to perform lymphadenectomy at the time of surgical treatment might be avoided, reducing the morbidity associated with this procedure.

Current recommendations suggest that patients with Stage Ib-II ISCC of the vulva should undergo radical excision of the tumour and bilateral inguinal lymphadenectomy or ipsilateral inguinal lymphadenectomy (if the lesion is lateralized to 1 cm or more from the midline of the vulva). Historically, reported morbidity has been high and relates in large part to wound dehiscence and lymphedema of the lower extremities, following inguinal lymphadenectomy. Impaired wound healing was found in 148 of 175 patients in one study, and postoperative lymphedema occurred in up to 69%; this was related to deep pelvic node dissection [2]. By limiting the nodal dissection to the inguinal nodes as well as the use of prophylactic antibiotic therapy, the incidence of significant lymphedema is thought to be much lower [28].

Recently it has been demonstrated that a variety of solid tumours contain hypoxic cells, theorized to result from neoplastic growth that exceeds its vascular supply, disorganized vasculature, and shunting of blood [29, 30]. Such cells represent a more aggressive phenotype, perhaps because a hypoxic micro-environment selects for cells that are genetically unstable and more likely to metastasise [7, 31-33]. One method for measuring hypoxic cells in clinical tumours is with the Eppendorf pO_2 electrode. With this instrument tumour hypoxia has been shown to be an independent predictor of treatment outcome in a variety of solid tumours [7-11]. Hence we hypothesized that tumour hypoxia might predict nodal metastasis which imparts a poorer prognosis in ISSC of the vulva.

Along with the oxygen electrode, other markers of tumour hypoxia exist, and require immunohistochemical assessment of tumour biopsies. As our hypothesis was that tumour hypoxia is associated with increased risk of nodal metastasis in ISCC of the vulva, we performed CAIX and MVD staining of the tumours.

Carbonic anhydrases catalyse the conversion of carbon dioxide to carbonic acid and are involved in a variety of biological functions. CAIX, a transmembrane glycoprotein, is a member of the carbonic anhydrase family. It may serve as a ligand or receptor in the regulation of intracellular communication, cellular proliferation or oncogenesis [34, 35]. CAIX is found in normal upper GI and a variety of GI related viscera but its over-expression has been associated with a poorer outcome in a number of solid tumours [12, 18-21]. In this current study, increased CAIX expression was not correlated with nodal spread but this may be due to the small numbers assessed.

Angiogenesis, a process by which new blood vessels proliferate from pre-existing vessels, is known to occur in solid tumours and is felt to be necessary in the neoplastic process [36]. Immunohistochemical staining that allows for measurement of microvessel density (MVD) in tumours is a method of assessing angiogenesis, and increased MVD has been associated with poorer prognosis in solid tumours [13-15, 17] including ISCC of the vulva [16]. In our series MVD did not correlate with nodal status. This may be due to the small number of patients as well as the widely distributed results in each

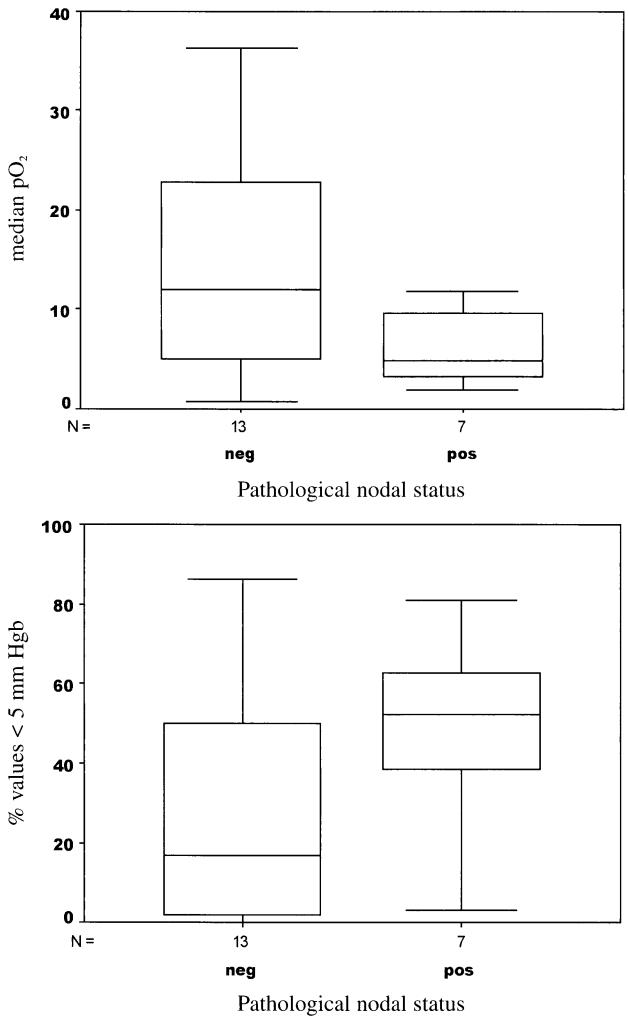

Figure 2. — Boxplot of Eppendorf measurements vs nodal status.

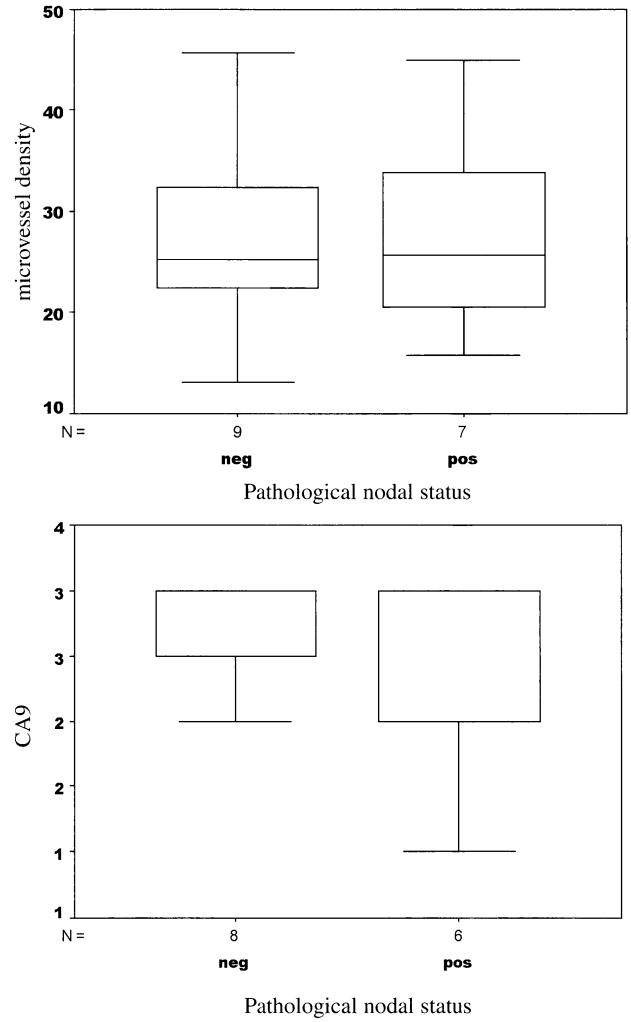

Figure 3. — Boxplot of a) MVD and b) CAIX vs nodal status.

nodal subgroup, both of which diminished the power of our analysis.

In this series MVD and CAIX were not correlated. Increased MVD was weakly correlated with tumour hypoxia measured as % values ≤ 5 mm Hgb ($p = 0.031$, $r^2 = .28$). As has been previously reported no correlation was found between CAIX and Eppendorf oxygen measurements [18]. In contrast to other reports, tumour size did not correlate with nodal status and is likely due to the small number of patients in our study [4].

Pretreatment anaemia in advanced cancers is an established prognostic factor. Anaemia may be due to local effects of chronic ongoing blood loss by the tumour, however with the median tumour size of 3.75 cm in our study group, it was not a significant clinical presenting complaint. The prognostic value in early cancer has been reported [37, 38]. It may be one manifestation of a systemic response to regional metastatic disease. In this small series low preoperative haemoglobin was associated with nodal metastasis. There was no significant correlation between Hgb status and either Eppendorf measurements or CAIX and MVD expression.

In conclusion, this small exploratory study has shown that pretreatment anaemia appears to be associated with nodal metastasis in ISCC of the vulva. Since 13 out of 20 subjects (65%) in this study underwent unnecessary lymph node dissections, future studies involving a larger group of patients is justified. Also, other markers of tumour aggressiveness need to be explored in an attempt to identify factors that would allow more selective criteria for performing lymph node dissection.

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