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The clinicopathological and microrna expression signature associated with lymphovascular invasion in squamous cell carcinoma: A basic descriptive study

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Abstract

Background and Aims: Lymphovascular invasion (LVI) is an indicator of lymph node metastasis and poor prognosis in various cancers including squamous cell carcinoma (SCC). Despite being easily resectable and having little potential for LVI; SCC displays aggressive behavior and often results in the death of the patient. With this in mind, it may be useful to investigate the clinical, pathological, and microRNA expression profile associated with LVI in SCC.

Methods: We evaluated the histological hallmarks associated with LVI from 16 formalin fixed paraffin embedded (FFPE) tissue samples (10 LVI−, 6 LVI+). We also quantified the expression of 10 microRNAs (hsa-miR-21-5p, hsa-miR-21-3p, hsa-miR-155-5p, hsa-miR-196a-5p, hsa-miR-375, hsa-let-7d-5p, hsa-miR-146b-3p, hsa-miR-221-5p, hsa-miR-205-5p, hsa-miR-491-5p), which have been previously identified to play a role in SCC development, using real time-PCR with the Qiagen miRCURY LNA SYBR Green PCR Kit.

Results: We observed a significant upregulation of microRNA-155, microRNA-196a, microRNA-375, and microRNA-221 in cases with lymphovascular invasion. Morphologically, we identified poor differentiation, dysplasia, loss of membrane polarity, high nuclear to cytoplasmic ratio, and the presence of squamous nests as defining features of LVI. Additionally, we found a gender bias and observed a tendency toward lymphatic invasion in lesions presenting around the perineal and abdominal regions.

Conclusion: We speculate that this profile may have prognostic significance and could guide the clinician in their treatment protocols for patients matching our genetic, demographic, and morphologic profile.

KEYWORDS

biomarkers, cutaneous, LVI, microRNA, SCC

1 | INTRODUCTION

Squamous cell carcinoma (SCC) is an invasive epithelial malignancy that arises from the squamous cell layers of the epidermis and shows keratinocytic differentiation. Risk factors include advanced age, sun exposure, fair skin, immunosuppression, and history of skin cancer.[1] SCC is capable of metastasis to regional lymph nodes which may be potentially lethal.[2] The risk factors for metastatic SCC include advanced Clark levels, tumor diameter ≥20 mm, extreme age, and location on the forehead or lip.[3]

Lymphovascular invasion (LVI) is described as the invasion of tumor cell into blood vessels or lymphatic system.[2] LVI is a primary and crucial phase in the systemic metastasis of cancer cells.[4] The presence of lymphovascular invasion is known to be a poor prognostic marker in various cancers including breast, urothelial and oral SCC.[5–10] In addition, the presence of LVI is associated with advanced tumor stage, high grades, lymph node metastasis in these cancers.[10–12] However, the molecular mechanisms mediating this prognostic feature remain undefined.

MicroRNAs are short, single-stranded, noncoding RNAs of approximately 22 nucleotides in length that regulate gene expression following transcription.[13] They do this by causing mRNA degradation or inhibiting translation. Determined by the pathways they regulate, microRNA in its mature form can function as either tumor suppressor or tumor promotor (onco-miRs).[13] To fully understand the role of microRNA in cancer progression, we must elucidate the process of microRNA production and the molecular mechanisms by which microRNA regulates gene expression.

Numerous microRNAs have been found to be dysregulated in SCC and there is sufficient evidence to suggest that their gene targets are involved in the multi-step process of carcinogenesis, as such, microRNAs have massive potential as biomarkers for invasive SCC.[14] The aim of the study was to determine the clinical, pathological, and microRNA expression profile associated with LVI in SCC.

2 | METHODOLOGY

2.1 | Ethics approval and consent to participate

Ethical clearance was obtained from the University of the Witwatersrand Human Research Ethics Committee (HREC) under M181093. In addition, waiver of informed consent for using human data was approved by the HREC.

2.2 | Sample selection and histologic review

A SNOMED search was carried out to identify the appropriate cases in the National Health Laboratory Services database. The following SNOMED term, M-80703 for "Squamous cell carcinoma, NOS" was used to identify cases diagnosed in 2016–2018. From the database search, 82 SCC cases were identified. Cases with reported LVI status, a tumor content of ≥80%, and available tissue block were included in the study. Sixteen cases were identified, six of which LVI positive, while the remaining 10 were LVI negative The slides and laboratory reports of the 16 cases were retrieved and blindly reviewed by a single pathologist for features associated with LVI.

2.3 | RNA extraction and complimentary DNA (cDNA) synthesis

The 16 FFPE blocks containing at least 80% tumor were selected for RNA extraction. These blocks were sectioned at 4 × 10 µm. Total RNA was extracted using the Qiagen RNeasy Kit for FFPE Tissue Sections as per the manufacturer's protocol (Qiagen). Xylene and ethanol were used to remove paraffin wax from tissues. Following deparaffinization the tissues were incubated with an optimized lysis buffer containing proteinase K. Elimination of genomic DNA was achieved by DNase treatment.

Finally, RNA was eluted with RNase-free water. To generate cDNA from the extracted RNA, miRCURY LNA RT Kit was used following manufacturer's protocol (Qiagen).

2.4 | Real-time PCR (RT-PCR)

The cDNA template was used to quantify miRNA gene expression using the miRCURY LNA miRNA PCR Assay (Qiagen). The reaction mix setup was performed according to manufacturer's reccomendations (Qiagen). The PCR cycling conditions included an initial heat activating step at 95°C for 2 min, 45 cycles of denaturation at 95°C for 10 s, followed by annealing and extension at 56°C for 60 s.

2.5 | Data analysis for RT-PCR

All data analysis was conducted using GeneGlobe Qiagen online software (https://geneglobe.qiagen.com/za/analyze). Raw data from the quantification step was exported into Excel and uploaded onto the GeneGlobe Data Analysis Center. The best housekeeping genes were selected using the "NormFinder" algorithm on Geneglobe. All six reference miRNAs were selected: *UniSp3*, *U6 snRNA (hsa, mmu)*, *SNORD48 (hsa)*, *UniSp6*, *SNORD44 (hsa)*, *has-miR-451a* for normalization using the delta delta Ct method.[15] *UniSp6 was used as* an internal amplification control. The Ct cut-off value for lower limit detection was set to 35. To compare the microRNA expression levels between the the test group (LVI+) and the control group (LVI-), an unpaired, two-tailed *t*-test was performed. A p value less than 0.05 was considered significant. A fold change value less than one, was considered a "downregulation," while a fold change value greater than one, "upregulation."

TABLE 1 The demographic and histopathological characteristics of the study cohort

	Total	LVI −	LVI +
Demographics			
Sample Size (*n*)	16	10	6
Mean age (years) (SD + / −)	56.12 (17.13)	61.30 (17.77)	47.50 (13.01)
Gender (F:M)	(1:1)	(2:3)	(2:1)
Site			
Wrist	1	1	0
Foot	1	1	0
Hand	1	1	0
Leg	3	3	0
Face	1	1	0
Scalp	1	1	0
Periauricular	1	0	1
Penis	2	1	1
Perianal	2	1	1
Abdomen	2	0	2
Cervix	1	0	1
Differentiation			
Poor	4	1	3
Moderate	11	7	4
Well	1	1	0
Ulceration			
Present	8	4	4
Absent	7	6	1
N/A	1	0	1
Dysplasia			
Present	13	9	4
Absent	2	1	1
N/A	1	0	1
Grade of dysplasia			
Mild	2	2	0
Moderate	1	1	0
Severe	10	7	3
N/A	3	1	2
Perineural invasion			
Present	2	0	2
Absent	14	10	4
Risk factor			
UV exposure	4	3	1
Oculocutaneous albinism	1	0	1
Previous non-healing trauma including burns and surgical scars	3	2	1
Immunocompromised individuals	3	2	1

Abbreviation: LVI, lymphovascular invasion.

3 | RESULTS

3.1 | Demographics and risk factors

The mean age at the time of SCC diagnosis across the sample was 56.12 (SD 17.13). Patients who were negative for LVI had a mean age of 61.30 (SD 17.77) and those who were positive for LVI had a mean age of 47.50 (SD 13.01) (Table 1). The sample had an equal number of men and women (1:1), from which 4/8 (50%) of women and only 2/8 (25%) of men developed LVI (Table 1).

Evidence of excessive UV light exposure was observed in 4/16 (25%) of patients, of whom 1/4 (25%) developed LVI. Oculocutaneous albinism was another significant risk factor in 1/16 (6.25%) of the patients, who subsequently tested positive for LVI. In cases where the patient had suffered previous cutaneous trauma that resisted healing 3/16 (18.8%), 1/3 (33.3%) developed LVI at that site. Finally, immunosuppression (HPV +, HIV+, ARV+) was recorded in 3/16 of the sample (18.8%), 1/3 (33.3%) of which had definitive LVI (Table 1).

3.2 | Site

A significant proportion, 37%, of SCCs developed on the extremities (wrists, feet, hands, and legs) of the patient, however, not one progressed to LVI. From the sample, 18.8% (3/16) of SCCs were found on the head and neck region (face, scalp, and periauricular) of these 33.33% (1/3) showed evidence of progression to LVI. Interestingly, 4/16 (25%) of SCCs were found in the perinium (penis and perianal) and in 50% (2/4) of those cases LVI was noted. Another 2/16 (12.5%) of SCC lesions developed on the abdomen, 100% of which became invasive. The cervical lesion, a non-cutaneous albeit squamous site composed only 1/16 (6.25%) of the case and showed complete invasion of the lymphatic system.

3.3 | Differentiation, ulceration, dysplasia, perineural invasion

Of the 4/16 tumors that were poorly differentiated (25%), (75%) showed extensive LVI. Comparatively, 1/1 (100%) of the well-differentiated subset, 1/16 (6.2%) did not progress to LVI. The greatest portion of the

sample 11/16 (68.8%) were moderately differentiated, from this group 36.4% (4/11) progressed to LVI while 63.6% (7/11) did not (Table 1).

More than a half 8/15 (53.3%) of cutaneous lesions were ulcerated, of which 4/8 (50%) were in the LVI negative group and 4/8 (50%) were in the LVI positive group. Of the cases without ulceration 7/15 (46.7%,) only 1/7 (14.3%) became involved in the lymphatics of the tissue.

Dysplasia was observed in 13/15 (86.66%) of cases, from this group 9/13 (69.2%) were negative for LVI, while 2/15 (13.3%) of the cases without any evidence of dysplasia, half (50%) were positive for LVI (Table 1). Not surprisingly, 10/13 (76.9%) of SCC cases exhibited severe dysplasia of which 3/10 (30%) showed LVI. In the "N/A" group the level of dysplasia could not be determined due to the extent of cutaneous destruction or absolute metastasis, from this subset 2/3 (66%) were positive for LVI.

Perineural invasion was present in only 2/16 (12.5%) of the sample however, 2/2 (100%) of those cases showed definitive progression to LVI.

3.4 | microRNAs upregulated in LVI+ cases

As reported in Table 2, overexpression of the following microRNAs were found in SCC test samples when compared to the control (LVI+ vs. LVI -); hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-196a-5p, hsa-miR-375, and hsa-miR-221-5p.

MicroRNA-221-5p had an FC = FR = 429.79, with the strongest significance score where $p < 0.01$ (Table 2). MicroRNA-375 had an FC = FR = 138.91 and $p < 0.01$. MicroRNA-196a-5p had an FC = FR = 27.25 and $p = 0.005$. MicroRNA-155-5p had an FC = FR = 8.5 and $p = 0.040$. MicroRNA-21-5p had an FC = FR = 2.06 however $p > 0.05$ indicated no significant association for the molecule between the two groups (Table 2).

TABLE 2 MicroRNA expression shown as Fold change (FC), fold regulation (FR), and *p* values from Qiagen

MicroRNA	Fold change (FC)	Fold regulation (FR)	*p* Value
SNORD48(hsa)	1.36	1.36	$p > 0.1$
hsa-miR-21-5p	**2.06**	**2.06**	$p > 0.1$
hsa-miR-155-5p	**8.50**	**8.50**	$\mathbf{p < 0.05}$
hsa-miR-196a-5p	**27.25**	**27.25**	$\mathbf{p < 0.01}$
hsa-miR-375	**138.91**	**138.91**	$\mathbf{p < 0.01}$
hsa-let-7d-5p	**0.43**	**−2.34**	$p > 0.1$
SNORD44(hsa)	**4.60**	**4.60**	$p > 0.1$
hsa-miR-451a	**0.39**	**−2.58**	$p > 0.1$
hsa-miR-146b-3p	1.30	1.30	$p > 0.1$
hsa-miR-21-3p	0.64	−1.56	$p > 0.1$
hsa-miR-221-5p	**429.79**	**429.79**	$\mathbf{p < 0.01}$
hsa-miR-205-5p	1.45	1.45	$p > 0.1$
hsa-miR-491-5p	0.75	−1.33	$p > 0.1$

Note: Values highlighted in bold show significant change.

3.5 | microRNAs downregulated in LVI+ cases

Decreased-expression of microRNAs hsa-let-7d and hsa-miR-491a were found when comparing the test and control groups (LVI+ vs. LVI-), though neither result could be confirmed as significant as shown in Table 2.

Let-7d had a decreased expression and showed FC = 0.43, FR = −2.34, however $p > 0.1$ which negates any significant association. Similarly, though microRNA-491a was downregulated with FC = 0.39 and FR = −2.58, $p > 0.1$ indicated no significant association in its relative expression.

4 | DISCUSSION

A demographic analysis found the mean age for SCC diagnosis to be 56.12 (SD 17.13), this does not coincide with the findings of the National Cancer Registry which finds the mean age for diagnosis to be between 60 and 70 years of age.[16] This could be attributed to the size of the sample for investigation which may not have been representative of the total population. However, it is worth considering that the available CANSA statistics are from 2017 and may themselves not be applicable in 2021. As such, we tentatively attribute the reduced age for SCC diagnosis to the collective effects of high levels of immunosupresion and harmful UV light radiation across the region, both of which are known to contribute to cancer progression.[17,18]

The mean age for a diagnosis of LVI in SCC from our sample was 47.50 (SD 13.01), this agreed well with other studies that found that if SCC were diagnosed before the age of 50 the patient had an increased risk for metastasis.[3] Despite the age related accumulation of driver and passenger mutations, younger patients are more likely to progress to LVI.[3] This highlights the need to find out why younger patients are more associated with LVI. We found that advanced age at the time of diagnosis had been shown to have relative contra-indications for surgical intervention in non-melanoma skin cancer of the face, suggesting that the metastatic potential of a cutaneous neoplasm could be determined by the genetic predisposition and environmental exposures specific to the patient as opposed to the gradual accumulation of driver and passenger mutations.[3,19]

A interesting demographic finding in Table 1 is the higher percentage of women than men with SCC positive for LVI. This may be due to the specific sites of SCC in this cohort that were positive for LVI, were from mucosal sites which are associated with HPV infection.[20] With regard to site-specific occurence, the largest portion of lesions in our sample were found on the extremeties (wrists, feet, hands, and legs), of which not even one showed LVI. Conversely, a significant portion of abdominal and perineal lesions did show evidence of LVI.

Risk factors specific to a South African context have been demonstrated to play a role in the progression of SCC.[21] Significant periods of exposure to ultraviolet light were thought to be the most common risk factor in the development of LVI as reported in other studies.[22,23] However, previous trauma to the site as well as immunosupression appeared to be of more consequence in our study. This may be due to the photoprotective effects of greater

melanin content within the basal layers of the epidermis in black South Africans, a georgraphy specific consideration.[17] This points to the endemic occurence of immunosupression across the population and highlights the need for adjusted interventions focusing on immune health hand-in-hand with to sun-screen application and covering exposed skin.[24,25]

With regard to site-specific occurance, Table 1 showed that the largest portion of lesions in our sample were found on the extremeties (wrists, feet, hands, and legs), of which not even one showed LVI. Conversely, a significant portion of abdominal and perineal lesions did show evidence of LVI. We attributed this to the finer points of gross anatomy, namely the larger lymphatic supply to the skin on the abdomen and the perineal strucutres when compared to the more distant and slowly draining extremeties (Mikhael & Khan, 2021).

Histopathologic analysis found poorly-differentiated tumors in the sample to have the greatest association with LVI, concuring with a well established clinical concept.[10,18] In agreement with Fania et al.,[1] ulceration and dysplasia were also dominant features across the sample with severe dysplasia found most frequently in LVI positive cases. Though underrepresented in the sample, perineural invasion showed absolute correlation with LVI. While abosolute correlation in hard to come by in studies with more than 16 samples, Fania et al.[1] also found PNI to be associated with progression from in-situ to invasive carcinoma.[1]

In developing a profile for LVI in SCC, we aimed to determine the 'hallmarks' of SCC in our sample by means of the light microscope in conjunction with careful evalutaion of our supporting data we found the dominating features of LVI in SCC to be; poor differentiation status, full thickness dysplasia, loss of membrane polarity, high nucelar to cytoplasmic ratio, and the presence of squamous nests. This is in line with LVI being a poor prognostic marker in SCC from various sites[10–12,26] and cancer types.[5–7]

The significant increased expression of microRNAs hsa-miR-155-5p, hsa-miR-196a-5p, and hsa-miR-221-5p were consistent with the available literature.[27–30] MiR-155 has been shown to predict poor prognosis and is associated with increase tumor size, advanced stage and increase depth of tumor invasion ([31,32] p. 155). MicroRNA 221 has been shown to promote SCC progression by targeting PTEN expression.[28] MicroRNA 196a has also been shown to be a biomarker of poor prognosis in cancers.[33] All in all, our results are supportive of previous data which suggest these microRNA to be upregulated in cases with poor prognosis, inclusive of cases expressing LVI.

However, the apparent upregulation of hsa-miR-375 was unexpected. Previous studies have shown a downregulation of this microRNA in SCC from various sites.[34–36] This may be attributed to the multiple functions a microRNA may posses, acting either as tumor suppressor or oncogene depending on the tumor[37,38]

4.1 | Limitations

This study was limited by small sample size and the lack of treatment and clinical outcomes.

5 | CONCLUSION

The study found an increased expression microRNA-155, microRNA-196, microRNA-375, and microRNA-221 in LVI+ cases. In addition, we identifed poor differentiation status, full thickness dysplasia, loss of membrane polarity, high nuclear to cytoplasmic ratios, and the presence of squamous nests to be associated with LVI in our cohort of SCC.

This profile generated in this study may assist in identifying high-risk patients and might highlight potential treatment regiments.

AUTHOR CONTRIBUTIONS

Shayene Robison: Conceptualization; data curation; formal analysis; writing – original draft. **Sharol Ngwenya**: Data curation; formal analysis; supervision; visualization. **Mulalo Molaudzi**: Conceptualization; Funding acquisition; Supervision; Writing – review and editing. **Julitha Molepo**: Conceptualization; funding acquisition; supervision; writing – review and editing. **Henry Adeola**: Conceptualization; supervision; writing – review and editing. **Pumza Magangane**: Conceptualization; data curation; formal analysis; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

TRANSPARENCY STATEMENT

The lead author Pumza Magangane affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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