Multidrug-resistance proteins and glucose transporter expression in cutaneous squamous cell carcinoma and adjacent healthy tissue in hairless mice

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Abstract. The present study examined the expression and potential roles of multi-drug resistance P-glycoprotein (P-gp), multi-drug resistance-associated protein (MRP) and glucose transporter type 1 (GLUT-1) in cutaneous squamous cell carcinoma (cSCC). Despite their recognized involvement in drug resistance, the specific contributions of these transporters to cSCC remain poorly understood. Using a cSCC hairless mouse model and through immunochemical analysis, the expression of P-gp, MRP-1 and GLUT-1 in cancerous and normal skin tissues was examined. The findings revealed that all assessed transporters were expressed across all skin tissues; however, the expression levels were notably higher in tumor and tumor-adjacent areas compared with normal tissues. This differential expression pattern suggests a complex interaction between these transporters and cSCC development, highlighting the nuanced nature of protein expression variations in cancerous and surrounding tissues. Despite the limited sample size, statistically significant variations in expression levels were observed. These findings may serve as a basis for hypothesis generation regarding the molecular mechanisms involved in cSCC development and progression, laying the groundwork for future investigations.

Introduction

Non-melanoma skin cancers are the predominant types of skin cancer, encompassing basal cell carcinoma and squamous cell carcinoma (SCC) (1). Cutaneous SCC (cSCC) is identified as the second most common skin cancer, next to basal cell carcinoma (2). It develops from precursory lesions known as actinic keratosis and has the potential to metastasize. The principal risk factor for cSCC is exposure to ultraviolet (UV) solar radiation, where total lifetime exposure plays a crucial role (3). The primary treatment method is surgical removal, although radiation therapy may be considered for certain patients (4). Individuals with weakened immune systems face an increased risk of developing cSCC, and while metastasis is uncommon in areas exposed to the sun, it is a more critical concern for those who are immunocompromised (5).

Within the field of cSCC research, mouse models are invaluable for investigating the origins and metastatic capabilities of the disease, as well as responses to treatments (6). Specifically, the dual-stage carcinogenesis protocol, which involves the use of 7,12-dimethylbenz(*a*)anthracene (DMBA) followed by 12-O-tetradecanoylphorbol-13-acetate, has been crucial in understanding tumor development and identifying genetic mutations commonly associated with cSCC (7).

Drug resistance in carcinomas can arise from numerous mechanisms, many of which remain unclear. A notable portion of these resistance processes is linked to the multidrug resistance (MDR) phenotype, which is facilitated by drug efflux pumps. These pumps are part of the ATP-binding cassette (ABC) protein family. P-glycoprotein (P-gp), encoded by the MDR1 gene, was the initial ABC transporter discovered to contribute to MDR (8). On the other hand, the multidrug resistance-associated protein (MRP-1), encoded by the ABCC-1 gene on chromosome 16p13.1, exhibits high expression levels in some types of tumors compared to healthy tissues (9). Additionally, cell lines with elevated expression levels of MRP-1 have exhibited resistance to several anticancer drugs, including doxorubicin, vincristine, etoposide and cisplatin (10).

Recent advances in the systemic treatment of advanced-stage cSCC have notably reshaped the therapeutic landscape, including the use of immune checkpoint inhibitors and targeted therapies (11-15). These therapeutic advances underscore the need for a more in-depth understanding of the molecular underpinnings of cSCC progression, particularly in relation to drug resistance mechanisms, such as

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those involving ABC transporters and glucose metabolism. It is considered that older cytotoxic regimens (e.g., platinum-based chemotherapy) exhibited limited efficacy and marked toxicity (16). The recent targeted therapies, including EGFR inhibitors, and particularly immune checkpoint inhibitors,such as cemiplimab and pembrolizumab, have demonstrated improved objective response rates and survival benefits, often with better tolerability. The neoadjuvant use of these agents is also emerging as a promising approach in high-risk resectable cSCC (17).

In this evolving systemic treatment scenario, understanding molecular pathways involved in tumor metabolism and drug resistance, such as the expression of glucose transporter 1 (GLUT-1), MRP-1 and P-gp, remains essential. Such insights could inform therapeutic stratification, identify biomarkers of response or resistance, and support the rational combination of local and systemic therapies.

GLUT-1, a key member of this family (GLUT protein family), plays a critical role in tumor cells by modulating glycolysis and cells' glucose uptake. Several studies have consistently demonstrated that GLUT-1 overexpression, which is linked to an increased glucose metabolism, is prevalent in various human solid tumors (18-21). In the majority of cases, GLUT-1 overexpression is associated with greater tumor aggressiveness and poorer clinical outcomes. Inhibiting GLUT-1, through reduced glycolysis and glucose uptake rates, leads to cell-cycle arrest and inhibition of cancer cell growth both *in vitro* and *in vivo* (22). Moreover, GLUT-1 overexpression has been linked to resistance to radiotherapy and worse outcomes in SCC (23).

As regards the analysis of the markers, P-gp, MRP-1 and GLUT-1, in relation to the diagnosis of cSCC, there are minimal data available. Specifically, for GLUT-1, one study on skin SCC reported that its expression was associated with the degree of cancer differentiation (18). This observation aligns with other data pertaining to oral SCC (24,25). A high expression of GLUT-1 and GLUT-3 in oral SCC appears to be linked to a poor prognosis. To the best of our knowledge, no study to date has specifically addressed the role of P-gp in the diagnosis of cSCC; however, in conjunction with the assessment of other parameters, it may enhance the diagnosis of oral SCC and appears to have relevant prognostic potential in esophageal SCC (26,27).

As regards MRP-1, beyond its undisputed contribution to resistance to therapy, no prognostic capability has been found for cutaneous, or other types of SCC. Moreover, no prognostic capability in relation to the diagnosis of SCC was traced concerning the combination of P-gp, MRP-1 and GLUT-1.

In the present study, in a hairless murine model of induced SCC of the skin, the expression levels of the markers P-gp, MRP1, and GLUT-1 were determined in cutaneous SCC and in the adjacent UV-irradiated and distant non-irradiated skin serving as a control. The aim of the present study was to observe these parameters and assess their relative diagnostic and prognostic value in cutaneous SCC.

Materials and methods

Reagents. DMBA and acetone, both exceeding 95% purity, were sourced from MilliporeSigma.

Animals. A total of 5 SKH-hr1 type hairless mice, aged 12 to 15 weeks and with an average weight of 26.87±1.93 g, were used to examine the induction of cutaneous SCC and the estimation of marker expression. These mice were obtained from the breeding facilities of the Section of Pharmaceutical Technology, Small Animal Laboratory, National and Kapodistrian University of Athens (Athens, Greece), holding a European License Code of EL 25 BIO-BR 06. The care provided to these animals was in strict compliance with the guidelines set forth by the European Council Directive 2010/63/EU. The mice had unlimited access to solid pellets (Keramaris Brothers & Co., General Partnership, Athens, Greece) and fresh water.

The mice were housed in an environment where the temperature was maintained at 24±1˚C and the humidity at 40±10%. A 1-week acclimatization period was allowed prior to the initiation of the experiments. The lighting regimen consisted of alternating 12 h of light and 12 h of darkness, using fluorescent lamps specifically chosen to emit no measurable UV radiation.

The experimental protocol received approval from the National Peripheral Veterinary Authority's Animal Ethics Committee, under the license no. K/4243/18-10-02. All procedures were performed in compliance with the ARRIVE Guidelines. Following the induction of carcinogenesis, the animals were euthanized by cervical dislocation according to the AMVA guidelines and skin samples were obtained.

UV exposure. A Xenon lamp of 1,000 W (model 6269) housed in an Arc Lamp Housing (model 66020-M) and powered by a Universal Power Supply (model 68820) from Oriel Instruments provided solar-simulated UV radiation (290-400 nm). This radiation was filtered through wavelength-specific filters, and the irradiance was verified prior to each experiment using a Goldilux Smart Meter (model 70239) from Oriel Instruments.

Induction of skin carcinogenesis by DMBA and UV radiation. The method for initiating skin carcinogenesis with DMBA and promoting it with UVR followed previously reported procedures (7). In brief, the back of each mouse received five topical applications of DMBA solution (0.015% in acetone), 0.2 ml each, at 5-day intervals. Following the final DMBA treatment, the mice were subjected to UVA (8.5 mW/cm^2) and UVB (8 mW/cm^2) radiation for 5 weeks, with 5 sessions per week. The initial exposure was set to a median erythemal dose (MED) of 32 mJ/cm^2 (7), with a 25% increase each subsequent week to skip skin thickening.

Skin sampling and homogenization. From each animal, three distinct skin samples were obtained: One cancerous (cSCC), one healthy, but adjacent to the cSCC (distance of ~1-2 mm), and one healthy, but distant (distance of ~30 mm). Following their classification, all samples were weighed and subsequently homogenized along with a 0.05% Tween-20 solution in PBS using a pyrex glass 21x150 mm homogenizer from Corning Inc.

Fluorometric measurements. To ensure assay reliability and reproducibility, several quality control measures were implemented throughout the immunochemical protocol. All

antibodies, both primary [GLUT-1 A-4 (sc-377228), MRP-1 QCRL-1 (sc-18835) and P-gp G3PP/P-GP E-10 (sc-390883)], as well the secondary antibody (mouse anti-rabbit IgG-CFL 488: sc-516248), were purchased from Santa Cruz Biotechnology, Inc. with established specificity. Phosphate-buffered saline (PBS) was provided by MilliporeSigma, while Tween-20 was supplied by ICN Biomedicals, Inc. MaxiSorb 96-well plates were purchased by Nalge Nunc (Thermo Fisher Scientific, Inc.). The primary antibodies were immobilized on high-affinity Maxisorb 96-well plates at a standardized dilution (1:250), under controlled pH conditions (carbonate buffer, pH 9.6) and temperature (4˚C for 12-16 h).

Following each incubation step, the wells were rigorously washed six times with PBS containing 0.05% Tween-20 to remove non-specifically bound material. The secondary antibodies used included fluorescently labeled anti-IgG conjugates. The secondary antibodies used included fluorescently labeled anti-IgG conjugates (mouse anti-rabbit IgG-CFL 488), applied at a dilution of 1:400, enabling quantification through fluorescence emission.

Fluorescence measurements were performed using the Fluostar Galaxy fluorometer, with excitation and emission wavelengths set at 485 and 520 nm, respectively. Each sample was normalized for both background signal (blank) and total protein concentration (mg/ml) using the following formula: A=(S-B)/Cs, where S is the signal from the sample, B is the corresponding blank value, and Cs is the sample protein concentration. This normalization produced values in relative fluorescence units (RFUs, ml/mg), enabling consistent comparisons across samples.

All samples were processed under uniform conditions using the same batches of reagents and standardized incubation protocols. This approach ensured intra-assay consistency and minimized procedural variability.

In brief, from each animal, three distinct skin samples were measured. Maxisorb plates were coated with 100 μl primary antibody, one for each protein, dissolved in a carbonate buffer solution (pH 9.6) at a 1:250 ratio. The preparations were sealed with parafilm and stored at 4˚C for a period of 12-16 h. Subsequently, the excess of the primary antibody solution was removed, and following six washes with 300 μl of a 0.05% Tween-20 in PBS solution, 100 μl of homogenized tissue sample was added and incubated for 30 min at room temperature, followed by six further washes and the addition of 100 μl of the secondary antibody suspension, and was subsequently allowed to stand at room temperature for a further 30 min. The plates were then washed six more times before incubating with 100 μl of the previously described fluorescein-tagged secondary antibodies for 15 min. After removing the contents of the wells and performing four consecutive washes, 100 μl washing solution (PBS, MilliporeSigma) was added to each well. The plates were then placed in a fluorometric device for the corresponding measurements. During these measurements, the emission filter of the device was set to 520 nm, while the excitation filter was adjusted to 485 nm. The resulting values are expressed in RFU/tissue mg.

Statistical analysis. Within-subject differences across three tissue types were analyzed (cSCC tumor, adjacent and distant) using a non-parametric repeated-measures approach (Friedman test) given non-normal distributions in multiple outcomes. Post hoc pairwise comparisons were performed with the Durbin-Conover procedure. Global Friedman P-values and adjusted pairwise P-values are presented. Analyses were conducted in Jamovi Cloud (https://www.jamovi.org). All analyses and graphical representations were conducted using GraphPad Prism 8.4.2 (Dotmatics). A P-value <0.05 was considered to indicate a statistically significant difference. To assess the adequacy of the sample size, a post hoc statistical power analysis was conducted using G*Power (version 3.1.9.7). The effect sizes (Cohen's f) for each protein were calculated based on the mean and standard deviation values across the three tissue types (cancerous, adjacent and distant).

Results

Post hoc statistical power analysis. Post hoc (observed) power, computed for the global Friedman tests using Kendall's W as the effect-size proxy, indicated moderate-to-large effects (MRP-1, W~0.39; P-gp, W~0.52; GLUT-1, W~0.54). At α=0.05, the corresponding observed powers were ~0.41 (MRP-1), ~0.52 (P-gp) and ~0.54 (GLUT-1), reflecting limited sensitivity at n=5. Prospective calculations suggest that ~13 (MRP-1), n~10 (P-gp) and n~9 (GLUT-1) would be required to achieve ~80% power (k=3 repeated measures).

DMBA-UV-induced mouse skin carcinogenesis. The development of tumors observed in the present study aligns with findings from prior research involving hairless mice, where tumors initially manifest as actinic keratosis and then benign papillomas. These elements then evolve into more aggressive forms before culminating in squamous cell carcinomas, mirroring the progression pattern documented in earlier studies (28).

MRP-1 assay. As illustrated in Fig. 1, for MRP-1 the global Friedman test did not indicate differences across tissues (χ^2=3.89, df=2, P=0.143). In pairwise comparisons, we observed a trend toward higher expression in cancer vs. distant tissue (P_adj=0.075) and no difference for adjacent vs. distant (P_adj=0.100), without reaching statistical significance after correction for multiple testing. This suggests a possible modest elevation of MRP-1 in tumor-proximal tissues, particularly cancer vs. distant, that did not meet conventional significance, likely reflecting limited power in this small exploratory cohort (n=5).

P-gp assay. In the analysis of P-gp expression (Fig. 2), the Friedman test suggested an overall trend (χ^2=5.20, df=2, P=0.074; W=0.52). In adjusted pairwise comparisons, the expression was higher in adjacent than distant non-cancerous tissue (P_adj=0.021), whereas cancer vs. distant showed only a borderline trend (P_adj=0.076) and cancer vs. adjacent was not significant (P_adj=0.438). This pattern suggests peritumoral upregulation of P-gp within the tumor field (adjacent skin), while differences involving the tumor itself were less consistent, an effect that will require larger samples to verify.

GLUT-1 assay. The statistical analysis of GLUT-1 expression levels across different skin tissue types revealed noteworthy outcomes, as illustrated in Fig. 3. The global test also revealed

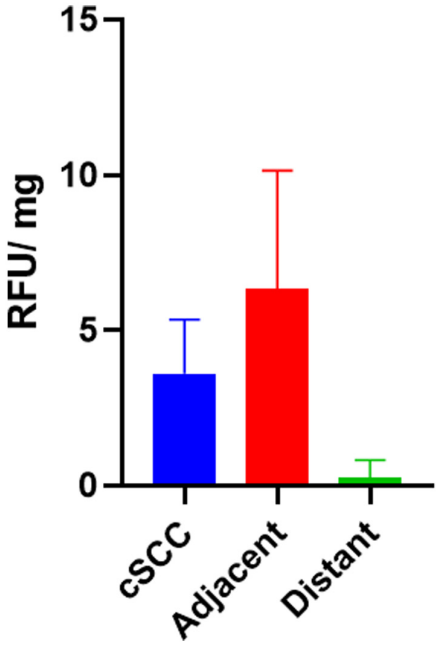

Figure 1. Multi-drug resistance-associated protein 1 measurements (n=5): Values are presented as the mean ± SD. Within-subject comparisons were made using the Friedman test with Durbin-Conover post hoc pairwise tests (adjusted P-values): global χ^2=3.89, P=0.143; cancer vs. adjacent P_adj=0.857; cancer vs. distant P_adj=0.075; adjacent vs. distant p_adj=0.100. No pairwise contrast reached statistical significance following adjustment.

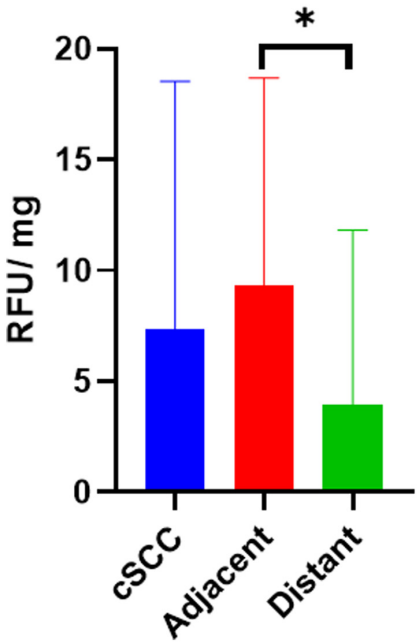

Figure 2. P-glycoprotein expression levels (n=5): Values are presented as the mean ± SD. Friedman test: global χ^2=5.20, P=0.074. Post hoc pairwise (adjusted): adjacent > distant *P_adj=0.021, cancer vs. distant P_adj=0.076 (borderline trend), cancer vs. adjacent P_adj=0.438. *P_adj<0.05.

a trend (χ^2=5.44, df=2, P=0.066; W=0.54). Post hoc tests indicated higher expression in cancer vs. distant (P=0.041), and adjacent vs. distant (P=0.021), whereas cancer vs., adjacent was not significant (P=0.670). These findings indicate that while GLUT-1 expression in the tumor itself is not markedly

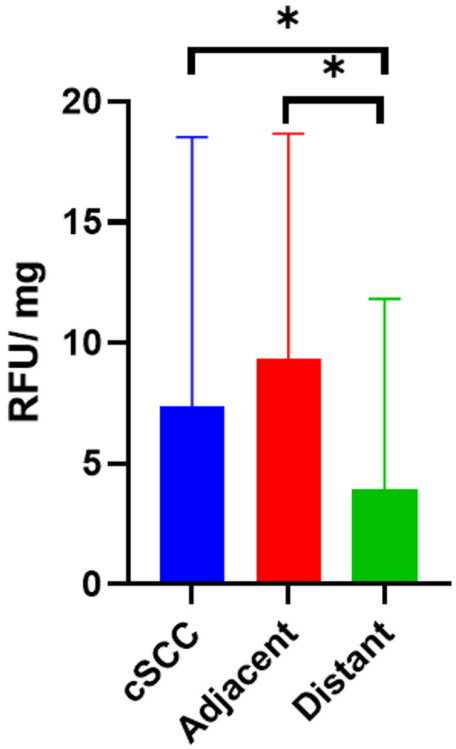

Figure 3. Glucose transporter 1 measurements (n=5): Values are presented as the mean ± SD. Friedman test: global χ^2=5.44, P=0.066. Post hoc pairwise (adjusted): cancer > distant *P_adj=0.041, adjacent > distant *P_adj=0.021, cancer vs. adjacent *P_adj=0.670. *P_adj<0.05.

different from surrounding or distant skin, perilesional tissue exhibits significantly higher GLUT-1 levels compared to distant normal skin, suggesting a metabolic adaptation in skin adjacent to the tumor.

Discussion

MRP-1, P-gp and GLUT-1 expression dynamics associated with cSCC revealed a complex interplay of statistical variances among different tissue groups. Notably, despite the relatively small sample size, certain statistically significant variances have emerged, rendering these findings particularly compelling. The post hoc power analysis (Friedman framework, effect size via Kendall's W) indicated moderate-to-large effects overall but limited sensitivity at n=5 (observed power ~0.41-0.54 at α=0.05). In line with this, pairwise differences reached statistical significance for GLUT-1 (cancer vs. distant; adjacent vs. distant) and for P-gp (adjacent vs. distant), whereas MRP-1 exhibited only a trend and did not reach significance. Thus, the lack of significance in some comparisons, particularly for MRP-1, may reflect insufficient power rather than absence of an effect. These observations merit further exploration within the context of cSCC development and its interaction with adjacent tissues. Especially considering that the elevated expression of P-gp, GLUT-1 and MRP-1 proteins observed has already been reported in the literature of squamous cell carcinomas (9,18,29,30).

A pivotal aspect of the analysis was the discovery that, progressing from the core site of cSCC towards the peripheral regions, there was an observable trend: The expression

of transporter proteins in adjacent normal tissues to cancer appears to be similar to that of cancerous tissues. However, in distant normal tissues from the cancer site, the expression is significantly reduced or even absent. The above model does not have strong statistical significance for all the transporters studied. Such variance underscores the distinct regulatory mechanisms and roles which these transporters play within the tumor microenvironment of cSCC, suggesting varied responses to oncogenic stimuli. Indeed, the expression of GLUT-1 has been reported to correlate with the various stages of squamous cell carcinoma (18).

Although the global Friedman test for P-gp did not reach conventional significance (χ^2=5.20, P=0.074), the adjusted pairwise comparison indicated higher expression in adjacent than distant non-cancerous skin (p_adj=0.021). The cancer vs. distant contrast exhibited only a borderline trend (p_adj=0.076), while cancer vs. adjacent was not significant (p_adj=0.438). Given the small sample (n=5), post hoc power under the Friedman framework was modest (observed power ~0.52 at α=0.05; Kendall's W≈0.52), indicating limited sensitivity to detect modest effects. Prospective calculations suggest that ~10 subjects would be required to achieve ~80% power for an effect of this magnitude. Larger cohorts are therefore warranted to determine whether the observed pattern reflects a true biological difference.

Despite the variations in protein expression relative to the distance from the cSCC site not uniformly reaching statistical significance, the consistent trend across all three studied proteins is notable. The findings suggest that the regions of healthy skin immediately adjacent to cSCC sites, which did not show signs of actinic keratosis, exhibited altered metabolic activity. This observation is pivotal, as it potentially unveils a precancerous alteration of the tissue that remains invisible to the naked eye, thereby identifying these regions as possibly being at an elevated risk for future oncogenic transformation. These results can provide a molecular-level perspective on the aggressive locoregional behavior of cSCC, observed locally as an extensive invasion into neighboring tissues (24).

This revelation bears notable clinical implications, particularly in the prognosis and therapeutic strategies for cSCC. It suggests the necessity of considering a broader therapeutic interest area around the tumor, acknowledging that the adjacent tissues, though not exhibiting the actinic keratosis phenotype, may still be undergoing critical, precancerous changes. Such an approach could fundamentally shift the paradigms of surgical or radiological interventions, advocating for the inclusion of a wider margin around the tumor to address areas with potential precancerous activity. This strategy aims to enhance treatment efficacy and possibly reduce the recurrence rates of cSCC, underscoring the critical importance of incorporating molecular and cellular insights into clinical decision-making processes.

The present study has several limitations which should be mentioned. Notably, the small sample (n=5 SKH-hr1 hairless mice) limits generalizability and increases the risk of type II error. While pairwise contrasts reached significance for GLUT-1 (cancer vs. distant; adjacent vs. distant) and for P-gp (adjacent vs. distant), MRP-1 did not reach significance and several comparisons only exhibited trends. A post hoc power assessment under the Friedman framework indicated modest observed power (~0.41-0.54 at α=0.05), and prospective calculations suggest that larger cohorts would be required, particularly for detecting modest effects in P-gp. The small sample also contributed to variability, as reflected in wide dispersions around the estimates. Accordingly, the findings should be interpreted as exploratory and warrant confirmation in larger studies with orthogonal validation.

Furthermore, no histological or immunohistochemical images were included to support the identification of cSCC and its distinction from adjacent or distant normal tissues. While the experimental model used is well established and tumor formation was confirmed macroscopically, the absence of microscopic validation using standard markers (such as Ki-67, CK5/6, p63 or p40) prevents a detailed morphological characterization of the lesions. Similarly, the lack of immunohistochemical localization of the target proteins (P-gp, MRP-1 and GLUT-1) limits the ability to correlate their expression with specific tissue structures or cell populations.

In addition, the methodology employed assessed total protein expression via a fluorometric solid-phase immunoassay, which, although appropriate for relative quantification, does not provide information about the functional activity of the transporters. Functional assays to measure efflux capacity or glucose uptake would be necessary to confirm the biological relevance of the observed expression levels. Similarly, complementary techniques, such as western blot analysis were not performed to validate the ELISA-based measurements.

The present study should therefore be considered an exploratory, hypothesis-generating study that provides preliminary insight into the expression gradients of drug-resistance and metabolic transporters in UV-induced cSCC. The model did not include any pharmacological treatment, and therefore reflects transporter expression under carcinogenic rather than therapeutic pressure. This is a critical distinction, as the expression and activity of proteins, such as P-gp are often modulated in response to chemotherapy.

Despite these limitations, the present study highlights biologically meaningful trends and suggests that membrane transporter expression in cSCC and its periphery may represent an early molecular shift relevant to carcinogenesis, with potential diagnostic or prognostic value. The present study also aimed to contribute to the growing body of research seeking to elucidate the molecular microenvironment of cSCC by demonstrating differential expression patterns of key membrane transporters not only within tumor tissue, but also in histologically normal-appearing adjacent skin. The findings presented herein underscore the presence of subclinical molecular alterations that may precede visible pathology.

The potential of this approach lies in its translational value. Following validation in larger preclinical and clinical cohorts, these transporters may serve as biomarkers for field cancerization, aiding clinicians in defining surgical margins more precisely or identifying areas of skin at high risk for malignant transformation. This highlights the concept of field cancerization, a process by which cumulative UV exposure induces molecular alterations in broader tissue zones, increasing susceptibility to malignancy (31,32). From a surgical perspective, while extending margins indiscriminately is often unfeasible due to anatomical or cosmetic constraints, our data may support more personalized surgical planning, especially in high-risk or recurrent cases.

Additionally, alternative therapeutic approaches could be explored. These include the application of targeted photodynamic therapy in the peritumoral zone (33), the use of topical metabolic inhibitors in clinically normal yet molecularly altered skin (34,35), or the implementation of molecular imaging tools, such as PET tracers targeting GLUT-1, to better delineate high-risk margins (36). While preliminary, such interventions could complement standard care by addressing subclinical disease spread. It is not exaggerated to assume that this field will evolve within the next 5 years, towards personalized cutaneous oncology, where local and systemic therapeutic decisions will be guided not only by histology but also by molecular and metabolic profiling. Research such as the present study aims to lay foundational research hypotheses for such approaches, by identifying early and subtle biological shifts at the tumor periphery.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GP and MCR were involved in the conception and design of the study. GP and MK performed the experiments, measurements and data collection. GP, CB, AV and MCR were involved in the analysis and interpretation of the results. AV drafted the manuscript. AV, CB, MCR reviewed the manuscript. All authors have read and approved the final version of the manuscript. GP, AV and MCR confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Mice were obtained from the breeding facilities of the Small Animal Laboratory at the School of Pharmacy, adhering to European License Code EL 25 BIO 06, in strict compliance with the guidelines outlined in European Council Directive 2010/63/EU. The procedures were conducted in accordance with the National Peripheral Veterinary Authority's Animal Ethics Committee license number K/4243/18-10-02 and adhered to the ARRIVE Guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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