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Cutaneous squamous cell carcinomas with markers of increased metastatic risk are associated with elevated numbers of neutrophils and/or granulocytic myeloid derived suppressor cells

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ABSTRACT

Background: A subset of presenting cutaneous squamous cell carcinomas (CSCC) is high risk with respect to their high rates of recurrence, metastasis and patient death. The identification of such high risk CSCC is problematic. Neutrophil and granulocytic myeloid derived suppressor cell (G-MDSC) numbers are elevated in a number of cancers, but their association with current markers of high risk tumors in the setting of CSCC is unknown.
Objectives: To assess circulating and tumor-localised neutrophil and G-MDSC populations for associations with high-risk tumor characteristics and overall survival (OS) in CSCC patients.
Methods: A retrospective clinical audit was performed of patients who had hospital operations for primary CSCC and did not have other malignancies or HIV. Therapeutically immuno-suppressed individuals (TII, n = 129) and non-TII (n = 29) were analysed separately with respect to the presence of high-risk tumor features and OS. In addition, 47 patients with prospectively collected blood and primary CSCC tumor samples were analysed to determine frequencies of circulating G-MDSC and tumor localised CD66b+ and CD8+ leukocytes.
Results: In the clinical audit of non-TII, high circulating neutrophil counts were associated with tumor thickness $\geq$ 5 mm, Clark level V and high T-stage. Univariate analysis showed elevated neutrophil count was a significant marker of poor OS, whilst tumor thickness remained the only independent histological predictor of OS after adjusting for age and immuno-suppression. The prospective study demonstrated that tumors $\geq$ 5 mm thick had significantly increased total and peri-tumorally localised CD66b+ leukocytes (comprising neutrophils and/or G-MDSC) and that elevated circulating G-MDSC numbers were associated with high T-stage tumors.
Conclusions: This study demonstrates that the presence of high risk CSCC is associated with increased numbers of both circulating and tumor resident populations of neutrophils and/or G-MDSC. These cell types therefore merit further investigation with respect to their functional and prognostic significance in CSCC.
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1. Introduction

Non-melanoma skin cancers, including cutaneous squamous cell carcinoma (CSCC), are the most common type of skin cancer worldwide, and in New Zealand the per capita mortality rates from skin cancer are among the highest in the world [1,2]. The majority of CSCC patients have an excellent prognosis following simple excision. However, a subset present with high-risk CSCC that are

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associated with high rates of recurrence, metastasis and disease specific death [3,4]. The immune system plays a critical role in the development of skin cancer and this is reflected in the elevated risk and aggressiveness of CSCC in immuno-suppressed individuals [3,5–7]. This high-risk subgroup of patients is therefore routinely targeted for increased clinical surveillance. However, within the wider population of apparently immuno-competent patients, the identification of high risk CSCC is problematic.

Currently, the most effective predictors of high-risk tumors are tumor thickness, [8–10] and the Brigham and Women's Hospital (BWH) tumor (T) staging system[11]. However, it is clear that a more effective risk stratification scheme is required to accurately identify high-risk CSCC [9,10,12], and there is currently considerable interest in defining additional markers of risk [11,13].

Neutrophils are a key immuno-modulatory population whose activities are thought to predominantly promote tumor progression [14]. This concept is supported by numerous studies that have associated increased circulating and/or tumor-infiltrating neutrophils with poor outcome in a range of cancers types including melanoma [14–17]. Studies in SCC have been limited to head and neck (HNSCC) and cervical SCC, where both neutrophil infiltration and elevated neutrophil/lymphocyte ratio have been associated with poor prognosis [18,19]. However, the relative numbers of circulating and tumor-infiltrating neutrophils in CSCC is presently unknown, as is their prognostic significance. Granulocytic myeloid derived suppressor cells (G-MDSC) share many of the features of neutrophils but represent a distinct immune-regulatory population [20] that has also been shown to be increased in various cancers [21,22]. We have previously documented the presence of elevated circulating G-MDSC numbers in some patients with CSCC [23], but their association with high risk tumors is unknown.

The significance of neutrophils and G-MDSC in the setting of CSCC is unknown. Analysing associations between the circulating and tumor-infiltrating numbers of these cells and clinical markers of high risk CSCC may clarify whether these cells have functional roles and/or prognostic value in CSCC. Therefore, in this study these potential associations were analysed using data from a clinical audit and prospectively collected patient samples.

2. Materials and methods

2.1. Clinical audit selection criteria

Records from the Department of Plastic and Reconstructive Surgery, Christchurch Hospital, New Zealand were searched to locate all individuals with excision of primary CSCC between January 1, 2009 and December 31, 2011, pre-operative complete blood cell counts (CBC) and comprehensive pathology reports, including all tumor characteristics (n = 332). Further data were retrieved from medical records, including survival time since diagnosis (date last seen or date of death).

Case exclusion criteria included *in situ* CSCC lesions, recurrent or metastatic CSCC, incomplete histopathology reports or medical records, and presence of diseases that modulate circulating leukocyte numbers (HIV, haematological malignancy, recent (<2 years) exposure to chemotherapy). Cases remaining were defined as the 'Initial Cohort' (n = 282).

Because a proportion of the patients presented with multiple tumors removed by one or more operations over a short period (<3 months), further selection criteria were applied to restrict case inclusion from that period to a single, representative CSCC tumor per patient. These tumors were identified as having, firstly, the highest BWH T stage (Supplementary Table 1, 2), and, secondly, the greatest tumor thickness. If a patient presented with a new primary CSCC more than three months after the previous CSCC, the tumor was included in the study as an independent incidence. Cases remaining after selection of representative tumors were defined as the 'Selected Cohort' (n = 168).

Cases in the Selected Cohort were subsequently divided into two groups based on whether or not patients were taking immunosuppressive medications (Prednisone, Azathioprine, Cyclosporine, Tacrolimus or Mycophenolate mofetil). Those taking such medications were defined as 'therapeutically immuno-suppressed individuals (TII; n = 129)' and those not taking such medications were defined as 'non-therapeutically immuno-suppressed individuals (non-TII; n = 39)'.

2.2. Prospective study of matched circulating and tumor-localised immune cell populations

Forty seven eligible non-TII (selection criteria as per 'Selected Cohort') donated pre-operative fresh blood and a primary CSCC tumor sample to the Cancer Society Tissue Bank Christchurch (CSTBC) in 2013. Patients provided informed, written consent for use of tissue and permission to access medical records. Ethical approval was granted by the Upper South Island Regional & University of Otago Human Ethics Committees. For each patient, a routine CBC was performed, and levels of G-MDSC were quantified by flow cytometry [24] using the gating strategy outlined in Supplementary Fig. 1.

Of the tumor samples in the prospective study, 25 were available for immuno-staining. Formalin fixed paraffin embedded tumor sections (2 μm) were prepared and immuno-stained as previously described [25]. Double immuno-labelling was performed manually according to the manufacturer's protocol using EnVision G|2 Doublestain System Rb/Mo DAB+/Permanent Red (Dako, Carpinteria, CA, USA). Primary antibodies were against the neutrophil, G-MDSC and eosinophil specific antigen CD66b (1:1000, clone GF10F5; BD Biosciences, San Diego, California) and the cytotoxic T-lymphocyte specific antigen CD8 (1:50, clone C8/144B, Dako). For antigen retrieval, slides were incubated in Tris-EDTA (pH 9.0) for 3 min in a pressure cooker, then cooled for 40 min at room temperature.

Immuno-histochemistry was evaluated by two independent observers (AM and AS), blinded to clinico-pathological information. The number of CD66b+ (neutrophils, G-MDSC and eosinophils) and CD8+ (cytotoxic T cells) stained cells observed in peritumoral and intra-tumoral locations were enumerated in five randomly selected high-powered fields (HPF, 400x, 0.23 mm^2) in areas with high immune cell infiltration (excluding areas of tissue necrosis and ulceration). Numbers of each cell type in each location were expressed as the average number of cells per HPF.

2.3. Statistical analysis

Categorical variables were compared by the Chi-squared and Fisher's exact test. Continuous variables between patient groups were compared using the Kruskal-Wallis test with Dunn's post-test and Mann Whitney *U* tests. Survival distributions were plotted using Kaplan-Meier plots and compared by the log rank test. Univariate and multivariate analysis of prognostic factors was carried out using the Cox proportional hazards regression model. Survival data for a New Zealand (NZ) population, with the same age and gender distribution as the patient cohort, were generated using life table data (years 2010–2012) from Statistics New Zealand [2]. These data were based on NZ birth and death registrations during 2000–2002. The Kaplan-Meier method was used for univariate analysis, and Cox regression (odds ratios with 95% CI) for multivariate analysis. All significance tests were two-sided, and p values of <0.05 were considered significant. Statistical analyses were performed using SPSS for Windows version 17 (SPSS Inc.,

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