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ANATOMICAL PATHOLOGY

Circumscribed sebaceous neoplasms: a morphological, immunohistochemical and molecular analysis

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Summary

Sebaceous neoplasms encompass a range of lesions, including benign entities such as sebaceous adenoma and sebaceoma, as well as sebaceous carcinoma. The distinction of sebaceous carcinoma from benign lesions relies on histological identification of architectural or cytological features of malignancy. In this study we have assessed the diagnostic discriminatory ability of mitotic rate and immunohistochemical markers (p53, bcl-2 and p16) in a selected group of well circumscribed sebaceous neoplasms, incorporating examples of sebaceous adenoma, sebaceoma and sebaceous carcinoma. We found that mitotic rate was significantly higher in malignant lesions as compared to benign lesions, but none of the immunohistochemical markers showed a discriminatory expression pattern. In addition, we performed a mutational analysis on the same group of lesions using next generation sequencing (NGS) technology. The most commonly mutated gene was *TP53*, although there was no correlation between the p53 immunohistochemical results and number or type of *TP53* mutation detected. *CDKN2A*, *EGFR*, *CTNNB1* and *KRAS* were also commonly mutated across all lesions. No particular gene, mutation profile or individual mutation could be identified which directly correlated with the consensus histological diagnosis. In conclusion, within this diagnostically challenging group of lesions, mitotic activity, but not immunohistochemical labelling for p16 or bcl-2, correlates with diagnostic category. While a number of genes potentially involved in the genesis of sebaceous neoplasia were uncovered, any molecular differences between the histological diagnostic categories remain unclear.

Key words: Sebaceous neoplasms; sebaceous carcinoma; sebaceoma; sebaceous adenoma; next generation sequencing.

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INTRODUCTION

Sebaceous neoplasms encompass a range of lesions, including benign entities such as sebaceous adenoma and sebaceoma, as well as sebaceous carcinoma. The distinction of sebaceous carcinoma from benign lesions relies on histological identification of architectural or cytological features of malignancy. While unequivocal infiltrative growth is generally readily recognised and suggests malignancy, well differentiated (low grade) sebaceous carcinomas may have a well circumscribed silhouette, and the distinction between these tumours and benign sebaceous neoplasia can be difficult. The criteria employed are often subjective and the thresholds at which a malignant interpretation is appropriate are not well defined. We have recently documented a significant degree of interobserver variation with regard to the diagnosis of circumscribed sebaceous lesions.[1]

The difficulties in separating the various types of sebaceous tumour are highlighted and perhaps magnified by controversies regarding their biological nature. Ackerman famously proposed that lesions conventionally considered to represent sebaceous adenoma are in fact a form of well differentiated sebaceous carcinoma,[2] while other authors have argued that they represent a form of intraepithelial sebaceous carcinoma.[3] Kazakov *et al.* described significant difficulty in classifying a small series of sebaceous neoplasms which displayed a 'benign' architecture but atypical cytology.[4] The term 'sebomatricoma' has been proposed as a designation for all benign sebaceous neoplasms to simplify classification.[5]

In an attempt to address the inherent diagnostic difficulties, several groups have assessed a number of antibodies for potential discriminatory value.[6–8] Taken together, these studies suggest that when compared with benign sebaceous neoplasms, carcinomas are characterised by increased expression of p53 and decreased expression of bcl-2 and p21. However, the applicability of data from these studies in cases of genuine diagnostic difficulty is limited by the inclusion of moderately and poorly differentiated sebaceous carcinomas.

In this study we have assessed the diagnostic discriminatory ability of several immunohistochemical markers (p53, bcl-2 and p16) in a selected group of well circumscribed sebaceous neoplasms, incorporating examples of sebaceous adenoma, sebaceoma and sebaceous carcinoma. Our aim in including only well circumscribed lesions was to assess the utility of these immunohistochemical markers in the group of diagnostically challenging lesions, where any discriminatory abilities would be most beneficial. Additional immunohistochemistry for mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 was performed on all cases and immunohistochemistry for β-catenin was performed on a subset of cases.

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In addition, we performed a mutational analysis on the same group of lesions using next generation sequencing (NGS) technology. As well as being the first comprehensive mutational analysis of sebaceous neoplasms to our knowledge, this also allowed us to compare the immunohistochemical findings for p53 and p16 with the mutational status of the underlying gene.

METHODS

Ethics

This study was approved by the Sir Charles Gairdner Hospital Research Ethics Committee.

Case selection

A total of 24 cases of sebaceous neoplasms were retrieved from the archives at PathWest, QEII Medical Centre. These cases were selected from a group of well circumscribed sebaceous lesions which had previously been used for an interobserver variability study.[1] As part of this study the cases had been reviewed by four dermatopathologists, and the 24 cases used in this investigation had a consensus diagnosis of sebaceous adenoma, sebaceoma or sebaceous carcinoma. A consensus diagnosis was defined as agreement between at least three of the four dermatopathologists. Of the 24 cases, 17 had agreement between all four of the dermatopathologists. The paraffin blocks were retrieved, and the cases were de-identified prior to analysis by assigning them a unique study number. Follow-up was via a combination of pathology record review, electronic medical record review and phone calls to the referring clinician. The average follow-up time was 7.5 years.

Mitotic counts

A mitotic count, recorded as mitotic figures per 10 high power fields (mf/10hpf, field diameter 0.55 mm) was obtained for each of the lesions by a single author (BAW).

Immunohistochemical analysis

All cases were stained for p16, p53, bcl2, MLH1, MSH2, MSH6 and PMS2, with a smaller subset of cases stained for β-catenin. All staining was performed using the Ventana BenchMark ULTRA platform (Roche, USA), with antibodies as outlined in Table 1. The immunohistochemical stains were interpreted by a single author (NTH). For p16 and bcl2 a visual estimation of the percentage of cells showing staining, as well as the intensity of staining (estimated on a 0–3 scale), were multiplied to create a staining score. For p53 only a percentage of positive cells was scored, based on work by Yemelyanova *et al.* demonstrating that the addition of an intensity measure did not improve the performance of this marker.[9] Only nuclear staining was considered positive for p53. For the mismatch repair proteins, retained nuclear staining for all four markers was required for a 'normal' designation, while the absence of any one of the four was considered to be an abnormal result.

Statistical analysis

Mitotic counts and immunohistochemical findings were compared between diagnostic categories using a Kruskal–Wallis test. Comparisons between the broader groupings of benign versus malignant were performed using a Mann–Whitney test. The number of mutations present in tumours with and without loss of mismatch repair proteins was also compared using a Mann–Whitney test. Non-parametric testing methods were chosen as we felt that the assumption of normality could not be upheld in these populations. The tests were performed using a publically available on-line calculator (http://vassarstats.net).

Next generation sequencing (NGS)

DNA extraction

A RM2125 RTS microtome (Leica Bioystems, Germany) was used to obtain two 10 µm sections from each FFPE tissue block for subsequent tissue digestion and DNA extraction. Representative sections (4 µm) obtained before and after tissue sectioning were stained with haematoxylin and eosin (H&E) to assess the proportion of tumour present in the final purified DNA sample. Prior to DNA extraction, the paraffin was first removed via a standard series of xylene and graded ethanol washes. DNA was extracted using a commercial magnetic bead separation method (ChargeSwitch gDNA Micro Tissue Kit; Life Technologies, USA), according to the manufacturer's instructions. The yield of purified genomic DNA was estimated using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Life Technologies) according to manufacturer's instructions.

Library preparation

The NGS platform used in this study was the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies). Library preparation for each sample was performed using the Ion AmpliSeq Library Kit 2.0 and Ion AmpliSeq Cancer HotSpot Panel v2 (Life Technologies) following the manufacturer's instructions. This targeted cancer panel sequences hot spot mutations from 50 oncogenes and tumour suppressor genes implicated in various cancers. Unique Ion Xpress Barcode Adapters 1-32 (Life Technologies) were ligated to the amplicons and subsequently purified using Agencourt AMPure XP Reagent (Beckman Coulter, USA). The amplicons underwent a second round of PCR amplification to complete their linkage with the adapters, with another purification step using Agencourt AMPure XP Reagent (Beckman Coulter). The amplified library was then quantified using the Qubit 2.0 Fluorometer (Life Technologies) according to the manufacturer's instructions. The final library concentrations were standardised to 100 pM in Ion AmpliSeq Low TE buffer (Life Technologies).

Emulsion PCR and semi-conductor sequencing

Ten uniquely barcoded library samples (100 pM each) were pooled. The final concentration of each pool was adjusted to 9 pM diluted in nuclease-free water, and then clonally amplified onto ion sphere particles (ISPs) by emulsion PCR with biotinylated primers using the Ion PGM Template OT2 Reagents 200 Kit (Life Technologies) and OneTouch 2 System (Life Technologies) according to manufacturer's instructions. Each pool was loaded onto an Ion 318v2 Chip (one pool per chip; Life Technologies) for single-end sequence analysis using the Ion PGM Sequencer using 500 flows (125 cycles) for 200-base-read-sequencing.

Coverage and data analysis

Data collected from the PGM were initially processed using the Ion Torrent platform-specific pipeline software Torrent Suite v3.6.2 to generate sequence reads and to filter and remove poor signal-profile reads. In particular this

Table 1 Details of the antibodies used for this study

Antibody	Manufacturer	Clone	Dilution
p16	Ventana	E6H4	PD
p53	Dako	DO-7	1:100
Bcl2	Dako	124	1:40
MLH1	Ventana	M1	PD
MSH2	Ventana	G219-1129	PD
MSH6	Ventana	44	PD
PMS2	Ventana	EPR3947	PD
β-catenin	Cell Marque	14	1:200

PD, pre-diluted from manufacturer.

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