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Detection of copy number variations in melanocytic lesions utilising array based comparative genomic hybridisation

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Summary

Distinction between melanocytic naevi and melanoma occasionally poses a diagnostic challenge in ambiguous cases showing overlapping histological features. Melanomas are characterised by the presence of multiple genomic copy number variants (CNVs), while this is not a feature of naevi. We assessed the feasibility and utility of array-based comparative genomic hybridisation (aCGH) to assess CNVs in melanocytic lesions. DNA was extracted from formalin fixed, paraffin embedded (FFPE) sections of unambiguous naevi ($n = 19$) and melanomas ($n = 19$). The test DNA and gender mismatched human reference DNA were differentially labelled with fluorophores. Equal quantities of the two DNA samples were mixed and co-hybridised to a SurePrint G3 Human CGH 8x60K array, and digitally scanned to capture and quantify the relative fluorescence intensities. The ratio of the fluorescence intensities was analysed by Cytogenomics software (Agilent). Frequent large CNVs were identified in 94.7% of melanoma samples, including losses of 9p (73.6%), 9q (52.6%), 10q (36.8%), 11q (36.8%), 3p (21%), and 10p (21%), and gains of 6p (42.1%), 7p (42.1%), 1q (36.8%), 8q (31.5%) and 20q (21%). Only one naevus showed two large copy number changes. Overall aCGH showed a specificity and sensitivity of 94.7% in separating naevi from melanomas. Based on our results, aCGH can be successfully used to analyse CNVs of melanocytic lesions utilising FFPE derived biopsy samples, providing a potentially useful adjunctive test for the classification of diagnostically challenging melanocytic proliferations.

Key words: Comparative genomic hybridisation; copy number variations; melanoma; melanocytic naevi; virtual karyotyping.

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INTRODUCTION

Distinction of melanocytic naevi from cutaneous melanoma by histopathological examination is a common component of the everyday workload for many pathologists. While morphological criteria for this separation are relatively well established, in practice these are not always easily applicable, and there are occasional ambiguous lesions characterised by overlapping features. This is reflected in several studies demonstrating only moderate interobserver agreement for the diagnosis of melanocytic lesions[1,2] while others have reported that up to 11% of melanocytic diagnoses are significantly changed after specialist review.[3]

Misdiagnosis can lead to significant under- or over-treatment, and a false negative diagnosis of cutaneous melanoma remains the most common reason for malpractice claims against pathologists.[4] Thus, there exists a clear need for ancillary testing to aid in the distinction between benign and malignant melanocytic proliferations in the setting of ambiguous histological features. The potential for cytogenetic aberrations to provide a diagnostic tool for separating melanoma from benign proliferations has been the subject of previous investigations.[5,6]

Gains and losses of genetic material are common in melanomas, but (with the exception of specific single abnormalities in Spitz naevi) not in melanocytic naevi.[7] Indeed melanoma is notorious for showing the most unstable genome amongst all human solid tumours.[8] Customised fluorescence *in situ* hybridisation (FISH) probes have been developed based on common chromosomal changes described in melanomas; however, these probes only assess limited loci and cannot interrogate the whole genome.[9] Array-based comparative genomic hybridisation (aCGH) can detect chromosomal gains and losses of variable size across the entire genome.[10]

Despite the attractiveness of aCGH as a diagnostic tool, its use in clinical practice is extremely limited, in part due to significant technical requirements in regard to assay optimisation and validation for formalin fixed, paraffin embedded (FFPE) tissue and the need for microdissection to enable isolation of relatively pure lesional DNA.[11] We assessed the feasibility and utility of aCGH in detection of CNVs in a cohort of unambiguous melanocytic lesions.

MATERIALS AND METHODS

Selection of cases

This study was approved as part of an intradepartmental routine quality and development exercise. A total of 45 samples of FFPE tissue reported as

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unambiguous malignant melanoma (MM) or melanocytic naevus were identified from the archives of Department of Anatomical Pathology, PathWest Laboratory Medicine (QEII Medical Centre, Perth, Western Australia). All cases were reviewed by two dermatopathologists (NMA and BAW) to confirm the original classification based on routine histological criteria. Two haematoxylin and eosin (H&E) stained slides representative of tumour blocks, one before and the other after 15 unstained slides, were examined and marked to select areas of viable tumour with maximum lesional cell content. These were then micro-dissected by scraping off 5 µm sections on glass slides using a scalpel blade. To be included in this study, lesions were required to have a minimum tumour thickness of 1 mm (using the Breslow methodology). Normal male and female genomic DNA (Promega, Australia) was used as reference DNA for array CGH experiments.

Genomic DNA extraction

Genomic DNA was extracted from FFPE tissue using the Qiagen DNeasy Blood and Tissue Kit, with modifications recommended by the ULS labelling system manufacturer (Agilent, Australia). Briefly, approximately 15 micro-dissected sections were heat deparaffinised at 90°C, followed by overnight treatment with 1 M sodium thiocyanate. This was followed by 48 h proteinase K treatment and then RNase A treatment. DNA was then purified using the Qiagen DNeasy Blood and Tissue Kit (Agilent), according to the manufacturer's instructions but substituting the wash buffer AW2 with 80% ethanol and eluting the DNA in nuclease-free water. Extracted DNA was quantified by spectrophotometry using a NanoDrop ND-2000 (NanoDrop, USA). Ratio of absorbance at 260/280 was used to assess DNA purity, and samples with a ratio of ~1.80 were regarded as sufficiently pure and suitable for ULS labelling. All DNA samples were visualised on 1.0% agarose gel and fragment sizes were assessed against a 1 kb DNA ladder.

DNA labelling

DNA was labelled using an optimised version of the protocol for ULS labelling of FFPE DNA (Agilent). Prior to labelling, reference and FFPE DNA was heat fragmented at 95°C for 10 and 2 min, respectively, then 250 ng of tumour and reference DNA was chemically labelled by incubating with 0.5 µL of ULS-Cy3 and Cy5, respectively, in a thermal cycler with a heated lid for 30 min. Un-reacted dye was removed using KREApure filters (Agilent). The degree of labelling (DoL) was determined according to the manufacturer's recommendations using the NanoDrop ND-2000. DoL values between 0.75% and 2.5% were regarded as optimal for Cy5, while values between 1.75% and 3.5% were optimal for Cy3-labelled DNA.

Array hybridisation and scanning

Cy3-labelled tumour DNA was combined with an equivalent amount of Cy5-labelled sex mismatched reference DNA. Repetitive sequences were blocked with human Cot-1 DNA (Invitrogen, USA) and samples were hybridised onto SurePrint G3 Human CGH Microarrays, 8x60K (Agilent) according to manufacturer's instructions. Following hybridisation for 40 h, microarray slides were washed according to manufacturer's instructions and stored under nitrogen before being scanned on a DNA Microarray Scanner (Agilent).

Data analysis

Scanned images were analysed using Feature Extraction software (Agilent), which normalises the fluorescent intensity of both dyes at each probe and calculates their ratio, expressed on a logarithmic scale (probe log2 ratio). It also computes a set of quality control (QC) metrics, including the average green and red signal intensity at all the probes as well as the background signal (noise) and signal-to-noise ratio using non-hybridising control probes. Feature extracted data were then analysed using CytoGenomics Software (Agilent).

FISH analysis

Five µm thick, unstained, FFPE tissue sections were subject to *in situ* hybridisation with melanoma four-colour FISH kit including probes for RREB1 (6p25), CEP 6 (6p11.1-q11.1), MYB (6q23), and CCND1 (11q13) as well as CDKN2A/CEP 9 FISH probe kit (Vysis, USA).[11,12] A standard hybridisation protocol was followed, as previously reported.[9] A minimum of 60 tumour nuclei were counted for FISH analysis.

SNP array analysis

Ten unstained sections from FFPE material were obtained for microdissection, with a single prior and subsequent H&E stained section used to confirm adequacy of tumour tissue and to mark areas for microdissection. DNA was extracted using a Qiagen extraction kit according to the manufacturer's instruction (Qiagen, Germany).

The Infinium HD assay was performed utilising Illumina iScan and HumanCytoSNP FFPE-12 BeadChip array according to the manufacturer's protocol (Illumina, USA). The BeadChips were stained, and then imaged, using a BeadArray Reader (Illumina). Image data were analysed with GenomeStudio (Illumina).

RESULTS

Bio-analytical adjustment

aCGH primary data were analysed according to the Aberration Detection Method 2 (ADM-2) algorithm as previously described.[10] The sensitivity threshold for the ADM-2 algorithm was adjusted based on self-self experiments performed on two samples. The post-analytic filters were also rectified based on two intra-array experiments. The optimal sensitivity threshold for the ADM-2 algorithm was defined as 6.1 after testing multiple thresholds. In addition, a minimum of 10 consecutive probes was established as the minimum post-analytic filter for CNVs which resulted in identical calls for intra-array duplicates performed on two samples. The log2 ratio threshold for detecting gains and losses of genomic material was set at 0.25.

Quality control (QC)

Opposite sex reference DNA was hybridised against each test DNA as an internal control for the quality and validity of results of each experiment. If the expected differences of the sex chromosomes (chromosome X and Y) were not met in a given sample, the experiment was considered unsatisfactory. QC metrics were also assessed for each sample. If QC metrics, in particular derivative log ratio (DLR) spread and red or green signal intensity, were not within the accepted limits, the experiment was considered unsatisfactory. A DLR spread of less than 0.5 was considered optimal, however DLR spread of equal or less than 0.6 was also accepted if the sex chromosome patterns were as expected. The samples with unsatisfactory results were repeated if there was adequate residual DNA or optimal remaining tissue to re-extract DNA.

Post-analytic interpretation

Copy number variants (CNVs) detected in each sample were first cross referenced with the Database of Genomic Variants (DGV) to exclude potential polymorphic CNVs.[13] Large CNVs involving at least 100 consecutive probes were defined as pathogenic if CNVs overlapped exonic regions. CNVs involving less than 100 consecutive probes were studied individually. Variants with high log2 ratio detected by Cytogenomic software as homozygous deletions or amplifications were also considered significant if overlapped with areas on the genome harbouring genes known to be involved in melanomagenesis such as *CCND1*, *CDKN2A*, *PTEN*, *P53*, *MYC*, *CDK4*, *NF1* and *MDM2*, or known to be frequently altered in melanomas such as *MYB1* and *RREB1*.[14] The remaining small (<100 probe) CNVs were considered as being of unknown significance.

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