ORIGINAL ARTICLE

Grading melanocytic dysplasia in paraffin wax embedded tissue by the nucleic acid index

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Background: Although nucleic acid derangements are the hallmark of melanocytic dysplasia, the gold standard for its diagnosis remains the microscopic evaluation of haematoxylin and eosin stained slides. However, light microscopy is subjective and crucial genomic changes do not always show as changes in histology.

Aims: To introduce the nucleic acid index (NAI) as a means of analysing nucleic acid derangements in histological sections at the level of the individual cell and within the context of its microenvironment.

Methods: Confocal laser scanning microscopy was performed on melanocytic lesions stained with acridine orange (AO), a fluorescent stain for DNA and RNA. The NAI, calculated by measuring the fluorescence intensities of AO in nuclei relative to the surrounding cytoplasm, reflects the concentration of DNA relative to RNA.

Results: When applied to benign naevi, dysplastic naevi, and melanoma, a very strong significant association was seen between lower NAI and malignant potential ($p < 0.0001$). Strong inverse correlations were found between NAI and both mitotic index and Breslow thickness. Interestingly, the NAI for dysplastic naevi is between that of melanoma and most benign naevi, consistent with their intermediate biological behaviour and histological appearance.

Conclusion: By providing a quantitative measure for melanocytic neoplasia, the NAI may improve the diagnosis of melanocytic lesions and the selection of treatment.

Melanoma, a highly aggressive disease that is increasing in incidence, is thought to arise via a series of mutations in melanocytes.^{1,2} The archetype for the development of this tumour is the familial atypical mole melanoma syndrome, wherein genetically unstable naevi (dysplastic melanocytic naevi; DMN) give rise to melanoma in situ, which then progresses to malignant melanoma.^{3,4} Transformation of melanocytes to a malignant phenotype may also occur in association with benign naevi, or even in the absence of evident precursor lesions.⁵

"It is crucial to diagnose melanoma accurately because early tumours can be cured by excision"

Melanocytic lesions may be difficult to classify and incorrect diagnoses are frequent causes of litigation.⁶ It is crucial to diagnose melanoma accurately because early tumours can be cured by excision. Diagnosing and grading DMN is also important because they are not only precursors to melanoma but are also markers for increased risk of melanoma.⁷ Although criteria for diagnosing dysplasia have been published, their application is notoriously subjective.^{8–10} For example, even among experts, there is only moderate concordance ($\kappa = 0.5$) in the grading of DMN.¹¹ Unfortunately, adjunctive tests (for example, MIB-1 index and morphometry) for discriminating melanocytic dysplasia rely on light microscopy and are themselves also subjective.¹²

One promising alternative to light microscopy is confocal laser scanning microscopy (CLSM), which combines high resolution with optical sectioning and the ability to use multilabelled probes. Although primarily used in cell biology, CLSM has been selectively applied to paraffin wax embedded tissue. For example, CLSM of breast and prostate lesions can distinguish between benign and malignant nuclei.^{13,14} However, these protocols are not practical for routine

pathology practice. Furthermore, these analyses were not quantitative and thus do not greatly improve upon the current gold standard of haematoxylin and eosin (H&E) microscopy. Unlike prostate and breast cancer, benign melanocytic lesions may exhibit subtle nuclear and architectural disorder, and there is even controversy as to whether nuclear changes alone can distinguish early dysplasia from benign precursors.⁹

Because abnormalities in nucleic acids are the cause of melanocytic dysplasia, genomic analysis of melanoma may aid in the diagnosis of difficult lesions. In fact, DNA has been studied extensively in melanoma tissue.¹⁵ However, genomic analysis requires extraction and pooling of a group of cells, thus losing information about individual cells and their histological distribution. In situ hybridisation, which can be performed in tissue sections, requires target specific probes and cannot assess global genetic changes. Technologies such as nuclear morphometry and argyrophilic staining of the nucleolar organising region have been used to study melanoma tissue; however, they are not amenable to routine use and provide only indirect evidence of DNA changes.^{12,16–19}

Cellular RNA concentrations positively correlate with tumour proliferative potential,^{20–23} but because of technical limitations, global RNA values have not been well studied in formalin fixed, paraffin wax embedded (FFPE) tumour tissue. The simultaneous quantitative analysis of both RNA and DNA at the level of the individual cell and within the context of its tissue microenvironment would be a powerful tool to study cancer. One potentially useful tool for such a study is the fluorescent nucleic acid dye acridine orange

Abbreviations: AO, acridine orange; CLSM, confocal laser scanning microscopy; DAPI, 4',6-diamidino-2-phenylindole; DMN, dysplastic melanocytic naevi; FFPE, formalin fixed, paraffin wax embedded; H&E, haematoxylin and eosin; NAI, nucleic acid index; PBS, phosphate buffered saline; SR, surface rendering

(AO), which has been used in benign FFPE tissues to label RNA and DNA.^{24–26}

In this context, CLSM was used to discriminate 28 benign, atypical, and malignant FFPE melanocytic lesions, each stained with AO and 4',6-diamidino-2-phenylindole (DAPI). The objective was to develop a quantitative index to aid in the diagnosis and treatment selection of melanocytic lesions. Our data show that the fluorescence intensity of AO in the nucleus relative to the cytoplasm (nucleic acid index) can distinguish between benign and malignant melanocytic lesions.

MATERIALS AND METHODS

Histological sections and slide preparation

Histological FFPE sections (5 µm thick) of benign and malignant melanocytic lesions were received as anonymised samples and with appropriate institutional ethical review board approval. H&E stained slides were reviewed for each specimen. The benign lesions included nine intradermal naevi and one compound naevus. In addition to one metastatic melanoma, we evaluated 12 primary melanomas and one melanoma in situ from all cutaneous sites, with a mean Breslow thickness of 2.3 mm (0.34–7.6 mm) and representing a wide range of cytologies and mitotic counts. Five dysplastic compound naevi were graded based on established criteria.^{8–10}

Unstained slides were dewaxed in xylene and rehydrated through descending grades of ethanol and eventually into water. The slides were incubated for five minutes in

phosphate buffered saline (PBS) supplemented with 0.2% Triton X-100, washed with PBS, and then stained with 1 µg/ml AO (Molecular Probes; Eugene, Oregon, USA) for 30 minutes in the dark. The slides were then washed three times with PBS for five minutes each, air dried, and coverslipped using DAPI containing mounting media (Molecular Probes).

Confocal analysis

The slides were examined with a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc, Thornwood, New York, USA) with an Axiovert 100M inverted microscope. For each digital image (512 × 512 pixels), approximately 45 optical slices were collected at 0.2 µm intervals using the Zeiss AIM software (v3.2) with a 100× 1.3 NA Zeiss Plan-Neofluor oil immersion objective. Using a multitrack configuration, the AO and DAPI signals were collected with a BP 505–550 filter and with a BP 385–470 filter after excitation with a 488 nm and 364 nm laser line, respectively. All settings were kept constant except for the detector gain, which was adjusted to just under saturation, thereby eliminating saturated pixels. Volume rendering and surface rendering (SR) were performed on images using Imaris software v4.0.3 (Bitplane AG, Basel, Switzerland) with a 5 µm grid. To determine the nucleic acid index, a representative 0.2 µm slice was selected from 10 benign naevi, five dysplastic naevi, 11 melanomas, and one melanoma in situ. Average fluorescent intensities of AO were measured in nuclei and the surrounding cytoplasm using the Zeiss LSM software. Three to six nuclei and surrounding cytoplasms were measured with regions of

Figure 1 Three dimensional surface renderings of melanoma stained with (A) acridine orange (green) and (B) DAPI (blue). (C) The images have been overlaid. (D) The same image was clipped along the Z axis to provide a side view of the tissue section. Note the mitotic figure (arrow). Images are projected on a 5 µm grid.

Figure 2 (A) Representative three dimensional surface renderings of benign naevi (1–3), dysplastic melanocytic naevi (4–6), melanoma in situ (7–8), and melanoma (9–12). Slides were stained with DAPI (blue) and acridine orange (green). A white bar through the equator of the DNA surface renderings (left panel) is included to illustrate the lack of symmetry in melanoma. (B, C) Measuring the nucleic acid index (NAI). The mean fluorescence intensities of acridine orange in a defined region of interest (white circles) of the nucleus (odd numbers) and surrounding cytoplasm (even numbers) were measured. The NAI was calculated by averaging the ratio of the nuclear to cytoplasmic intensities for three to six cells/slide. Representative optical sections of (B) benign naevus without detectable cytoplasm and (C) malignant melanoma.

interest of varying dimensions and shapes to determine the mean and standard deviation for each specimen.

RESULTS

FFPE tissue sections (5 μ m) were stained with two fluorescent nucleic acid dyes: AO and DAPI. DAPI, which binds to DNA, is concentrated in the nucleus. AO binds to RNA and DNA, thus staining the cytoplasm and the nucleus. Selective treatment with RNase and DNase showed that 90% of the nuclear staining with AO results from DNA, whereas cytoplasmic staining results from RNA.^{25–27} The stained sections were optically sectioned at 0.2 μ m intervals by CLSM and composite three dimensional SRs of RNA and DNA were calculated (fig 1). Rotation of this image provides a unique three dimensional side profile of the tissue section (fig 1D).

Three dimensional SRs of nucleic acids were calculated for a wide range of melanocytic lesions and evaluated by visual inspection as a function of their H&E based diagnosis. Melanoma was characterised by an irregular DNA shell with a heterogeneous distribution of nuclear DNA, resulting in a “Swiss cheese” appearance (fig 1B, 2A). DNA SRs from benign naevi were more uniform and displayed bilateral symmetry around an imaginary line drawn through their central long axis (fig 2A). Cytoplasmic RNA SRs of melanoma were more expansive and hierarchically branched relative to benign naevi (fig 2A). In fact, cytoplasmic AO staining was not even identified in seven of the 10 benign naevi (table 1).

As expected, five DMN had nucleic acid distributions that were more asymmetrical than benign naevi but not as irregular as melanoma. One example of melanoma in situ resembled that of invasive melanoma. However, visual inspection of SR is still subjective and does not improve on the gold standard H&E.

To develop a quantitative correlate for melanocytic atypia and malignant potential, the intensity of AO fluorescence was measured in representative 0.2 μ m optical sections from each lesion. Because measuring absolute nucleic acid values is not possible, a nucleic acid index (NAI) was calculated by measuring the fluorescence intensity of AO in the nucleus relative to the surrounding cytoplasm (fig 2B). As mentioned above, cytoplasmic AO correlates with RNA, whereas 90% of nuclear staining is the result of DNA. Therefore, the NAI closely parallels the ratio of DNA (nucleus) to RNA (cytoplasm). DAPI staining was used only to highlight the nuclear DNA and was not included in the quantitative analysis.

The NAI was analysed for one metastatic melanoma and 27 primary cutaneous melanocytic lesions, including benign naevi, dysplastic naevi, and melanoma (table 1). The mean NAI for melanoma was 2.0 (SD, 0.6). In contrast, cytoplasmic AO could not even be detected in seven of the benign naevi, precluding calculation of the NAI. In the remaining three naevi, the mean NAI was 4.4 (SD, 0.8). By H&E, these last three naevi had no distinguishing histological features relative to the other seven naevi. The NAI for dysplastic

Table 1 Nucleic acid index of melanocytic lesions

Mean (SD) NAI	Diagnosis, site	Breslow thickness	Mitotic index (/10 HPF)
1.2 (0.3)	Malignant melanoma, foot	7.6	8
1.4 (0.1)	Malignant melanoma, back	6	10
1.5 (0.2)	Malignant melanoma, eyebrow	4.0	10s
1.6 (0.5)	Malignant melanoma, back	2.6	30
1.6 (0.1)	Metastatic melanoma, axilla	N/D	N/D
1.7 (0.1)	Malignant melanoma, knee	7.0	5
1.7 (0.2)	Melanoma in situ, ear	N/D	N/D
2.2 (0.3)	Malignant melanoma, ankle	0.53	5
2.2 (0.4)	Malignant melanoma, head	2.0	5
2.7 (0.2)	Malignant melanoma, back	4.4	3
2.7 (0.7)	Malignant melanoma, chest	1.87	5*
2.7 (0.3)	Malignant melanoma, breast	0.34	5*
2.8 (0.6)	Malignant melanoma, back	1.2	5
2.9 (0.5)	Dysplastic naevus, abdomen	N/D	N/D
3.5 (0.4)	Benign naevus, head	N/D	N/D
3.6 (0.5)	Dysplastic naevus, groin	N/D	N/D
4.2 (0.3)	Dysplastic naevus, breast	N/D	N/D
4.3 (0.5)	Benign naevus, neck	N/D	N/D
4.6 (0.3)	Dysplastic naevus, not provided	N/D	N/D
5.1 (0.5)	Benign naevus, axilla	N/D	N/D
5.8 (0.8)	Dysplastic naevus, back	N/D	N/D
N/D	Seven benign naevi	N/D	N/D

*Estimated because the lesion occupied only 2 HPF.
HPF, high power field; NAI, nucleic acid index; N/D, not determined.

naevi ranged from 2.9 to 5.8. The NAI value did not correlate with cell size, biopsy procedure, or body location (table 1 and data not shown).

Even excluding the seven benign samples with no detectable cytoplasmic staining, significant correlations were found between the NAI and the three categories of lesion: benign naevi, DMN, and melanoma. By an exact Jonckheere-Terpstra trend test,²⁸ the two tailed p value for the comparison between NAI and the three groups (benign, dysplastic, and malignant), was 2.2×10^{-5} (or $p < 0.0001$), confirming a consistent trend between decreasing NAI and benign to dysplastic to malignant lesions. Thus, even based on available data (without the added benefit of the seven benign naevi), there was a highly significant association between NAI and benign, dysplastic, and malignant categories. By Spearman (non-parametric) correlation analysis of the 11 primary melanomas, strong inverse correlations were found between NAI and Breslow thickness (-0.70 ; $p = 0.016$) and between NAI and mitotic index (-0.76 ; $p = 0.0062$).

DISCUSSION

Melanoma arises via dysplasia of cutaneous melanocytes, either in the context of a pre-existing naevus or de novo. The histology of a melanocytic lesion (for example, DMN) cannot always be correlated with its biological potential, and incorrect diagnoses are not infrequently rendered. Moreover, microscopic evaluation of H&E stained tissue sections is inherently subjective, contributing to frequent discordant diagnoses. The NAI was developed as a quantitative tool to improve the accuracy and diagnostic reproducibility of difficult melanocytic lesions.

“Because the nucleic acid index does not rely on subjective evaluation, it should be superior to current adjunctive tests that rely on scoring by a pathologist”

Because melanocytic dysplasia is driven by derangements in nucleic acids, we developed this technology to study nucleic acid abnormalities in FFPE tissue at the level of the individual cell. In fact, a highly significant association was found between lower NAI and malignant potential ($p < 0.0001$). Because the NAI does not rely on subjective

evaluation, it should be superior to current adjunctive tests that rely on scoring by a pathologist.

The NAI is a quantitative estimate of the ratio of cellular DNA to RNA. Previous studies have shown that total RNA values are raised as a function of the tumour proliferation rate.^{20–23} Thus, the NAI provides a quantitative correlate for the proliferative potential of melanocytic lesions. Indeed, strong inverse correlations were found between NAI and both mitotic index and Breslow thickness. NAI may be helpful in the correct diagnosis of borderline lesions without identifiable mitotic bodies or when only limited tissue is available for study. A larger study is required to determine whether the NAI is an independent prognostic factor.

DMN represent a biologically and histologically intermediate stage of dysplasia. In addition to DMN being a precursor to melanoma, the grade of the melanocytic dysplasia correlates with the risk of developing melanoma.⁷ Because light microscopy is inherently subjective, there is a need for a quantitative assay to diagnose and grade these difficult lesions. In a small sample of DMN, the calculated NAI was intermediate between that of benign naevus and melanoma. Interestingly, whereas 70% of the benign naevi had no detectable cytoplasmic AO staining, three had NAI values in a similar range to dysplastic naevi. It is tempting to speculate that these three naevi may have a biological potential or predictive value similar to that of DMN. Alternatively, a low NAI may reflect the previous observations that a subset of benign naevi may harbour mutations in Ras or B-Raf.^{29–30}

Take home messages

- We have developed a method, the nucleic acid index (NAI), that can quantitatively measure melanocytic neoplasia
- The NAI could provide an alternative/adjunct to light microscopy, which has the disadvantage of being subjective and that crucial genomic changes do not always show as changes in histology
- The NAI may improve the diagnosis of melanocytic lesions and the selection of treatment

These results are intriguing and warrant additional study in an attempt to develop quantitative criteria that better reflect biological potential. A quantitative index will improve interobserver reliability and aid in clinical studies of DMN progression and risk of melanoma.

This protocol was designed with the practising pathologist in mind. Routine histological slides of 5 µm thick sections of formalin fixed, paraffin wax embedded tissues were used. Slide preparation included a single 30 minute incubation step. This technology may easily be applied to study unstained slides from archives and community based practices.

NAI may reduce the interobserver variability inherent in evaluating pigmented lesions by microscopy. Apart from the requirement of a confocal laser scanning microscope and three dimensional SR software, the processing involved a simple 30 minute stain of archived FFPE 5 µm tissue sections. We are currently analysing a series of atypical and unusual pigmented lesions to develop objective criteria that may provide clinically relevant information for those lesions that are not easily classified by H&E.

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