INVESTIGATIVE REPORT

Quantitative Analysis of Formaldehyde-induced Fluorescence in Paraffin-embedded Specimens of Malignant Melanomas and Other Melanocytic Lesions

Hiroyuki HARA[1], Michiko NAITO[1], Tomonori HARADA[1], Isao TSUBOI[1], Tadashi TERUI[2] and Shin AIZAWA[1]
[1]Division of Anatomical Science, Department of Functional Morphology, [2]Division of Cutaneous Science, Department of Dermatology, Nihon University School of Medicine, Tokyo, Japan

Inter-observer agreement is problematic in the histopathological diagnosis of melanoma and melanocytic naevi, even among expert pathologists. Formaldehyde-induced fluorescence (FIF) has been used for histochemical demonstration of catecholamines, 5-hydroxytryptamine and their immediate precursors. FIF can detect melanogenic activity and may be useful in differentiating malignant melanoma from other melanocytic lesions. The fluorescence of various types of melanocytic lesions has been previously studied quantitatively in formalin-fixed and paraffin-embedded sections. This study compared 2 sets of excitation and emission bands: 450–490 nm excitation/510–560 nm absorption filters (filter unit A) and 480 nm excitation/<510 nm absorption filters (filter unit B). Higher FIF was observed with filter unit A than with filter unit B. FIF intensity of central regions was found to be higher than that of the peripheral regions. Mean FIF was significantly higher in malignant melanomas than in naevi. Fluorescence imaging with filter unit A gave better diagnostic performance. In conclusion, quantitative measurement of FIF is a useful marker of malignant potential. *Key words: formaldehyde-induced fluorescence; digital fluorescence microscope; quantitative study; melanocytic lesions.*

Hiroyuki Hara, Division of Anatomical Science, Department of Functional Morphology, Nihon University School of Medicine, 30-1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173-0032, Japan. E-mail: hara.hiroyuki@nihon-u.ac.jp

Malignant melanoma is a highly aggressive tumour and its incidence rates have been increasing worldwide (1). Early detection and correct diagnosis is crucially important in order to decrease mortality (2). However, it is often difficult to determine histopathologically whether melanocytic lesions are benign or malignant. Inter-observer agreement is problematic in the histopathological diagnosis of melanoma and melanocytic naevi, even among expert pathologists (3). In a previous study, 8 panel members reviewed specimens of melanomas and melanocytic naevi, including Clark naevi and Spitz naevi,

and 38% of interpretations were discordant (3). Shoo et al. (4) reported that the discordance rate for diagnosis of melanomas and melanocytic naevi among pathologists was approximately 15%.

The formaldehyde-induced fluorescence (FIF) method has been used as a highly sensitive and specific method for the histochemical demonstration of catecholamines, 5-hydroxytryptamine (and their immediate precursors), L-DOPA and 5-hydroxytryptophan, at the cellular level (5–7). Observation of formalin-fixed paraffin-embedded (FFPE) unstained specimens using a fluorescence microscope is considered to be a simple and valuable method for proving melanogenic activities and for differentiating malignant melanoma from other melanocytic lesions (8–12). However, the results based on subjective visual assessment of FIF have not been sufficiently convincing to attract wider interest (2). Recent development of digital imaging makes it possible to quantitatively measure the intensity of FIF. To our knowledge, only 2 reports have been published regarding quantitative measurements of FIF (2, 13). The aims of the current study were: (*i*) to measure the intensity of FIF using 2 sets of excitation and emission bands; and (*ii*) to investigate the possibility of distinguishing melanomas from other benign pigmented lesions, based on FIF intensities.

MATERIALS AND METHODS

Skin samples, which were completely excised, were obtained from 31 cases of superficial spreading melanoma (SSM), 19 cases of nodular melanoma (NM), 30 cases of Clark naevus, 19 cases of Spitz naevus, 13 cases of Reed naevus and 30 common melanocytic naevi (CMN). The study was approved by the ethics committee at the Nihon University School of Medicine. Specimens were routinely fixed in 10% formalin and embedded in paraffin. Sections 3-μm thick were deparaffinized and unstained sections were mounted with the Vectabond™ reagent (Vector Laboratories Inc., Burlingame, CA, USA).

Fluorescence imaging

The sections were examined for fluorescence and the images were recorded at ×100 magnification using a BioZero-8000 microscope (Keyence, Osaka, Japan), equipped with 450–490 nm excitation/510–560 nm absorption filters (filter unit A) (Optp Science Inc., Tokyo, Japan) and 480 nm excitation/<510

nm absorption filters (filter unit B) (Keyence, Osaka, Japan). Fluorescence intensity measurements were analysed using digital image analysis software VH-H1A5 (Keyence, Osaka, Japan). FIF intensities of the central and peripheral regions of the tumour nests were measured separately. The area of strongest fluorescence in each region was selected for quantitative measurement.

Statistical analyses

Non-parametric statistical tests were used for comparison of the 6 different diagnostic groups. The statistical significance of the FIF intensity was calculated using the Kruskal-Wallis H-test with a *p*-value of less than 0.05, while the Friedmann multiple comparison procedure was performed for separate comparison of individual diagnostic groups. Receiver operating characteristics (ROC) analyses were used to assess diagnostic performance. The area under the ROC curve (AUC) was investigated. AUC was calculated as the proportion of the area of the entire graph that was beneath the curve (14). The AUC is expected to be 0.5 in the absence of predictive capability, whereas it is expected to be 1.00 in the case of high predictive capability (15). ROC curves can also be used to display the relationship between sensitivity (true-positive rate, y-axes) and 1-specificity (false-positive rate, x-axes). Statistical analysis was performed using SPSS version 22.0 (IBM, S & I Co. Ltd, Japan) and ystat2013.xls (Igaku Tosho Shuppan, Japan).

RESULTS

Fluorescence imaging with a 450–490-nm excitation filter and a 510–560-nm absorption filter (filter unit A)

Fluorescence imaging of all cases using filter unit A showed the presence of yellow to yellow-green fluorescence, which was observed for the nests and individual cells both of malignant melanomas and other melanocytic lesions. Fluorescence was observed in the nucleolus, the nuclear membrane, the cytoplasm and the cell membrane (Fig. 1A). This fluorescence disappeared after 2 min treatment with 0.1% borohydride in 80% isopropanol (data not shown). It was therefore considered that this fluorescence was specific and was due to the presence of reacting monoamines. The fluorescence intensity in the central regions of the lesions was relatively higher than that in the peripheral regions of the lesions. Some heavily pigmented cells were observed to show brown-orange fluorescence, as described previously (11). The fluorescence was also seen in the surrounding keratinocytes and cornified layer, especially in melanomas and Clark naevi. Tumour cells in the epidermis and upper dermis showed higher fluorescence intensity, while lower fluorescence intensity was observed in the middle dermis.

The median value, standard deviation (SD), standard error (SE) and *p*-values of the intensity of the fluorescence for the 6 diagnostic groups in the central regions are given in Fig. 1B and Table SI[1]. The median value increased considerably from benign to malignant lesions. When the diagnostic groups were analysed separately, the strongest fluorescence intensity was observed in SSMs, followed by NMs and Clark naevi. Among naevi, the fluorescence intensity of Clark naevi was relatively high, sometimes even higher than the fluorescence intensity that was determined for SSMs and NMs. The lowest fluorescence intensity was emitted by CMNs. There were significant differences between the fluorescence intensity
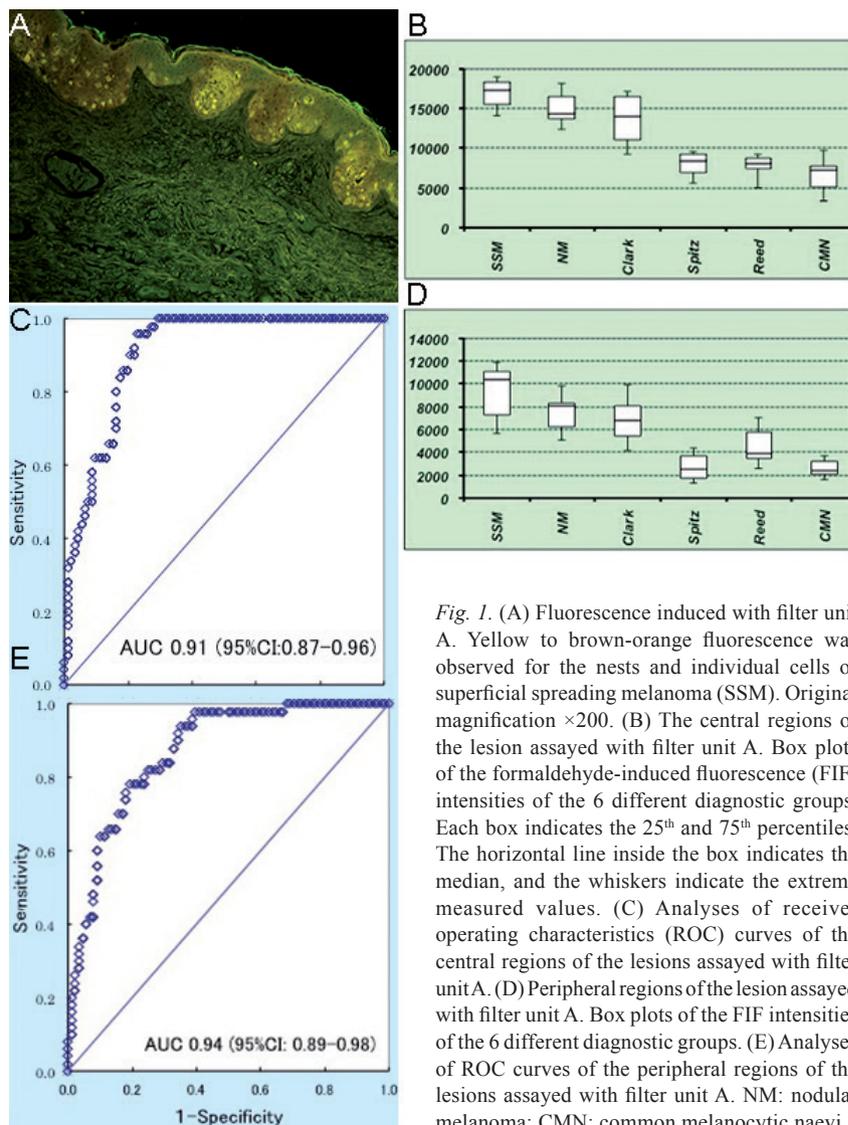
Fig. 1. (A) Fluorescence induced with filter unit A. Yellow to brown-orange fluorescence was observed for the nests and individual cells of superficial spreading melanoma (SSM). Original magnification ×200. (B) The central regions of the lesion assayed with filter unit A. Box plots of the formaldehyde-induced fluorescence (FIF) intensities of the 6 different diagnostic groups. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values. (C) Analyses of receiver operating characteristics (ROC) curves of the central regions of the lesions assayed with filter unit A. (D) Peripheral regions of the lesion assayed with filter unit A. Box plots of the FIF intensities of the 6 different diagnostic groups. (E) Analyses of ROC curves of the peripheral regions of the lesions assayed with filter unit A. NM: nodular melanoma; CMN: common melanocytic naevi.

of SSMs and Spitz naevi ($p<0.01$), of SSMs and Reed naevi ($p<0.01$), and of SSMs and CMNs ($p<0.01$). There were also significant differences between the fluorescence intensity of NMs and Spitz naevi ($p<0.01$), of NMs and Reed naevi ($p<0.01$), and of NMs and CMNs ($p<0.01$). No significant differences were observed between the fluorescence intensity of melanomas and Clark naevi. The fluorescence intensity of Clark naevi differed significantly from that of the other 3 diagnostic groups (Clark naevi vs. Spitz naevi: $p<0.01$, Clark naevi vs. Reed naevi: $p<0.05$, Clark naevi vs. CMNs: $p<0.01$).

ROC analyses of melanomas (SSMs and NMs) vs. other pigmented melanocytic naevi including Clark naevi were performed (Fig. 1C). Excellent diagnostic performance was achieved. The area under the ROC curve (AUC) was 0.91 (95% confidence interval [CI], 0.87–0.96). Sensitivity and specificity derived from the ROC curve were 0.88 and 0.82, respectively.

Fig. 1D and Table SI[1] show the results of the fluorescence intensities detected in the peripheral regions of the lesions. Overall, the results were relatively similar to those of the central regions. The median values of the FIF intensities were lower than those determined in the central regions for all diagnostic groups. There were significant differences between the fluorescence intensity of SSMs and of each of the naevi, excluding Clark naevi (SSMs vs. Spitz naevi: $p<0.01$, SSMs vs. Reed naevi: $p<0.01$ and SSMs vs. CMNs: $p<0.01$). There were significant differences between the fluorescence intensity of NMs and Spitz naevi ($p<0.01$), and of NMs and CMNs ($p<0.01$). No significant differences were observed between the fluorescence intensity of melanomas and Clark naevi. There were significant differences between the fluorescence intensity of Clark naevi and Spitz naevi ($p<0.01$), and of Clark naevi and CMNs ($p<0.01$).

The corresponding AUC was 0.94 (95% CI, 0.89–0.98) (Fig. 1E). The sensitivity and specificity for distinguishing melanomas from benign melanocytic lesions in the peripheral regions were 0.78 and 0.80, respectively.

Fluorescence imaging with a 480-nm excitation filter and a < 510-nm absorption filter (filter unit B)

All of the cases examined using filter unit B revealed green fluorescence (Fig. 2A). The median value, SD,
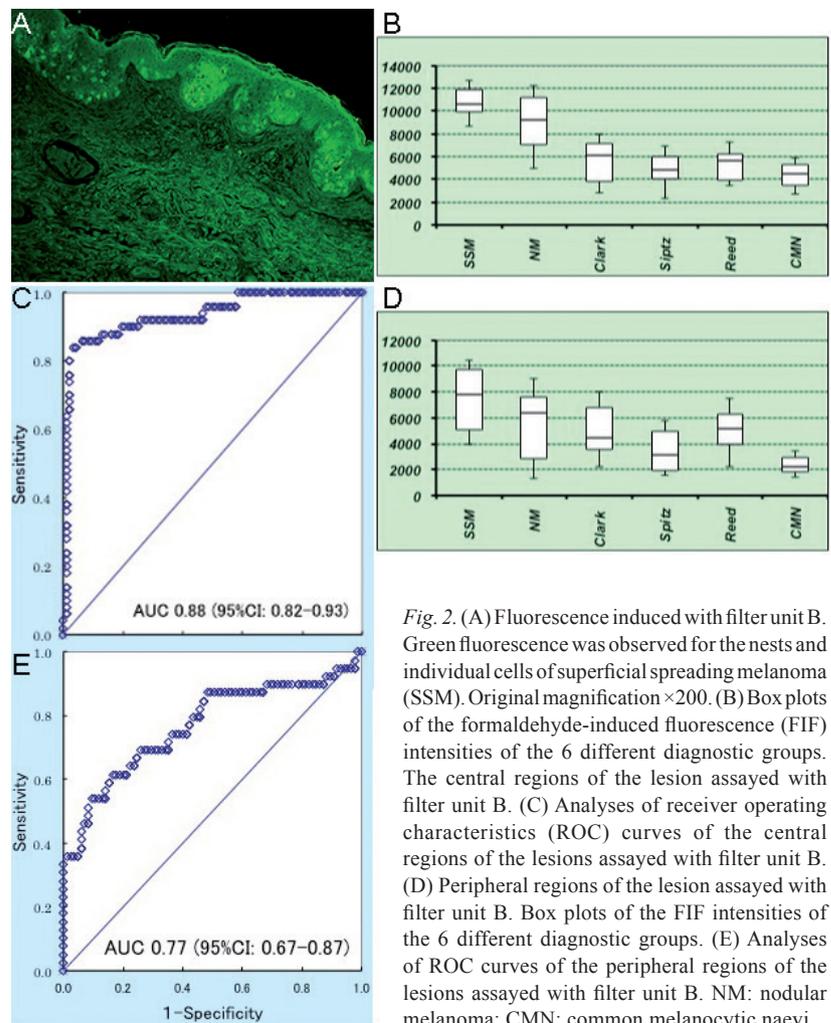

Fig. 2. (A) Fluorescence induced with filter unit B. Green fluorescence was observed for the nests and individual cells of superficial spreading melanoma (SSM). Original magnification ×200. (B) Box plots of the formaldehyde-induced fluorescence (FIF) intensities of the 6 different diagnostic groups. The central regions of the lesion assayed with filter unit B. (C) Analyses of receiver operating characteristics (ROC) curves of the central regions of the lesions assayed with filter unit B. (D) Peripheral regions of the lesion assayed with filter unit B. Box plots of the FIF intensities of the 6 different diagnostic groups. (E) Analyses of ROC curves of the peripheral regions of the lesions assayed with filter unit B. NM: nodular melanoma; CMN: common melanocytic naevi.

SE and p-values of the fluorescence intensity of the central regions of the lesions of the 6 diagnostic groups are given in Fig. 2B and Table SII[1]. There were significant differences between the fluorescence intensity of SSMs and that of each of the benign naevi ($p<0.01$). It was noteworthy that there were significant differences between the fluorescence intensity of SSMs and Clark naevi ($p<0.01$). There were significant differences between the fluorescence intensity of NMs and Spitz naevi ($p<0.01$) and of NMs and CMNs ($p<0.01$). The area under the ROC curve was 0.88 (95% CI, 0.82–0.93) (Fig. 2C). The sensitivity and specificity derived from the AUC in the peripheral regions of the lesions were 0.78 and 0.80, respectively.

The results for the peripheral regions were different from those of the central regions (Fig. 2D, Table SII[1]). There were significant differences between the fluorescence intensity of SSMs and that of each of the naevi excluding Clark naevi (SSMs vs. Spitz naevi: $p<0.01$ and SSMs vs. CMNs: $p<0.01$). There were no significant differences between the fluorescence intensity of NMs and that of any of the benign naevi. There were significant differences between the fluorescence inten-

sity of Clark naevi and CMNs ($p < 0.01$), and of Reed naevi and CMNs ($p < 0.05$). The area under the ROC curve was 0.77 (95% CI, 0.67–0.87) (Fig. 2E). The sensitivity and specificity of an attempt to distinguish melanomas from benign pigmented lesions were 0.69 and 0.68, respectively.

DISCUSSION

This study aimed to determine whether there were differences in the quantitative measurements of the FIF intensities between malignant melanomas and other melanocytic lesions. Observation of FFPE specimens obtained from melanocytic lesions has been carried out since 1982 (9). This method is not new (2); it is a simple and useful diagnostic tool for distinguishing malignant melanomas from other melanocytic lesions. The specific fluorescence emitted by melanoma cells and melanocytes is mainly due to the presence of 5-S-cysteinyldopa (5-S-CD), which is a precursor in the formation of pheomelanin (16). 5-S-CD has been used as a useful biochemical marker for detecting melanomas and for monitoring the clinical course of patients with melanoma (16–19). In general, the fluorescence intensity of naevus cells is less than that of melanoma cells. We previously analysed 5-S-CD values using high-performance liquid chromatography (HPLC) in tissues obtained from malignant melanomas and other pigmented skin lesions, and concluded that the pigmented lesions can be diagnosed as malignant melanoma when the 5-S-CD value in the tissue is greater than 100 ng/mg (20). FIF has not been widely used in spite of its usefulness (2). One reason for its lack of use is that different researchers use different spectral bands for excitation and emission. Chwirot's group has developed quantitative measurements of the intensity of FIF (13). In studies by this group, FIF intensity was expressed as a percentage value of the fluorescence intensity standard and was determined for all of the cells of the skin layers of interest within the lesion (2, 13). On the other hand, our system directly and quantitatively measured the FIF intensities by digital imaging. The present study compared 2 sets of excitation and emission bands and measured fluorescence intensity in the central and peripheral regions of the lesions. Generally, FIF intensities excited at 450–490 nm were higher than those excited at 480 nm. The fluorescence intensities in the central regions of the lesions were higher than those in the peripheral regions. It has been previously shown that fluorescence intensity depends on the distance of the fluorescent cells from the centre of the lesion (9–11, 13). In the present study, ROC analyses of the diagnostic value of the data were undertaken by comparing malignant melanomas and naevi. We were able to achieve excel-

lent diagnostic performance. AUC values between 0.6 and 0.7 are considered to indicate a weak diagnostic capability, values between 0.71 and 0.8, a satisfactory diagnostic capability and values greater than 0.8, a good diagnostic capability (21). Many cancer marker specific assays have good sensitivities/specificities, with AUCs of 0.8–0.9 (22). However, no quantitative data on diagnostic marker typically used for differential diagnostics of melanomas and melanocytic lesions are available (2). In the present study, a good diagnostic performance was observed for FIF, except for the fluorescence of the peripheral regions that were excited with 480 nm (AUC 0.77, 95% CI 0.67–0.87). From the ROC analyses, high sensitivities and specificities were obtained using the fluorescence data of the central regions emitted either with the 510–560 nm band or with the < 510 nm band. In particular, better diagnostic performance was obtained by fluorescence imaging with filter unit A. Consequently, ROC analyses indicated that quantitative fluorescence measurements could be used for diagnostic purposes, specifically for distinguishing melanomas from naevi.

Because FIF intensities in Clark naevi were sometimes higher than those in melanomas, the differences in FIF intensities between melanomas and Clark naevi were statistically insignificant in the present study. However, the mean fluorescence intensity determined for the whole regions of melanomas was higher than that of Clark naevi. Duncan et al. (23) reported that overall agreement in distinguishing Clark naevi from malignant melanomas and common melanocytic naevi was 77% among the 5 experienced dermatopathologists. FIF intensities in some melanomas were lower than those in benign melanocytic lesions (2). Clark naevi are known to be risk factors for malignant melanoma (24). Mammalian melanocytes produce 2 types of melanin, eumelanin (the black to brown pigment) and pheomelanin (the yellow to red melanin). Microanalytical methods to quantify the amounts of eumelanin and pheomelanin have been developed (25). Clark naevi lesions contained higher amounts of pheomelanin than other melanocytic naevi (26, 27). Recently, a multiphoton technique based on pump-probe spectroscopy was developed to determine the microscopic distribution of eumelanin and pheomelanin in pigmented lesions of human skin (28). Future work will aim at examination of pump-probe images for the discrimination of melanomas from Clark naevi.

In conclusion, FIF intensities may aid in the distinction between melanomas and melanocytic naevi. Quantitative fluorescence measurements are simple, reproducible and useful for discriminating malignant melanomas from benign melanocytic naevi. Further investigation will be needed in order to differentiate Clark naevi from malignant melanomas.

ACKNOWLEDGEMENTS

REFERENCES

1. Cancer facts and figures 2009. Atlanta: American Cancer Society, 2009.
2. Sztramska A, Piwinski M, Chwirot BW. Quantitative measurements of formalin-induced fluorescence for differential diagnostics of melanomas and lesions of human skin. Melanoma Res 2010; 20: 408–415.
3. Farmer ER, Gonin R, Hanna MP. Discordance in the histopathologic diagnosis of melanoma and melanocytic nevi between expert pathologists. Hum Pathol 1996; 27: 528–531.
4. Shoo BA, Sagebiel RW, Kashani-Sabet M. Discordance in the histopathologic diagnosis of melanoma at a melanoma referral center. J Am Acad Dermatol 2010; 62: 751–756.
5. Corrodi H, Jonsson G. The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. J Histochem Cytochem 1967; 15: 65–78.
6. Falck B, Hillarp N-Å, Thieme G, Torp A. Fluorescence of catechol amines and related compounds condensed with formaldehyde. J Histochem Cytochem 1962; 10: 348–354.
7. Morishima T. Histochemical studies on the monoaminergic nerve in the skin chiefly by means of the fluorescence method of Falck and Hillarp. Acta Derm Venereol 1970; 50: 329–337.
8. Temple-Camp CRE, Hainsworth M. The fluorescence of melanocytic lesions. Am J Dermatopathol 1986; 8: 290–294.
9. Inoshita T, Youngberg GA. Fluorescence of melanoma cells – a useful diagnostic tool. Am J Clin Pathol 1982; 78: 311–315.
10. Dalal BI, Slinger RP. Formaldehyde-induced fluorescence in melanomas and other lesions. Arch Pathol Lab Med 1985; 109: 551–554.
11. Shukawa T, Nonaka S, Yoshida H. A comparative study of fluorescence in malignant melanoma and nevocellular nevus using a fluorescence microscope and formalin-induced specimens. J Dermatol 1990; 17: 538–544.
12. Morishima C, Morishima T. Fluorescence microscopic findings of formalin-fixed paraffin-embedded unstained specimens of malignant melanoma, pigmented nevus, and allied diseases: a comparative study with immunostaining of the same specimens with HMB-45. Jpn J Dermatol 1996; 106: 119–128.
13. Chwirot BW, Sypniewska N, Swiatiak J. Diagnostic potential of fluorescence of formalin-fixed paraffin-embedded malignant melanoma and pigmented skin lesion. Quantitative study of fluorescence intensity using fluorescence microscope and digital imaging. Melanoma Res 2001; 11: 569–576.
14. Klingenstein A, Haritoglou I, Schaumberger MM, Nentwich MM, Hein R, Schaller UC. Receiver operating characteristic analysis: calculation for the marker 'melanoma inhibitory activity' in metastatic uveal melanoma patients. Melanoma Res 2011; 21: 352–358.
15. Hanley IA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982; 143: 29–36.
16. Morishima T, Tatsumi F, Fukuda E, Saito M, Fujita M, Nagashima N, et al. Cysteinyldopa isomers and DOPA in lesions and urine of Japanese patients with malignant melanoma. Arch Dermatol Res 1983; 275: 76–79.
17. Wimmer I, Meyer JC, Seifert B, Dummer R, Flace A, Burg G. Prognostic value of serum 5-S-cysteinyldopa for monitoring human metastatic melanoma during immunochemotherapy. Cancer Res 1997; 57: 5073–5076.
18. Meyer T, Hauschild A, Kromminga A, Hartleb J. Amdt R, Christophers E, et al. Clinical evaluation of 5-S-cysteinyldopa testing using a new and optimized system as a tumor marker of malignant melanoma. Melanoma Res 2002; 12: 471–477.
19. Wakamatsu K, Kageshita T, Furue M, Hatta N, Kiyohara Y, Nakayama J, et al. Evaluation of 5-S-cysteinyldopa as a marker of melanoma progression: 10 years' experience. Melanoma Res 2002; 12: 245–253.
20. Hara H, Chino K, Kawanami T, Sameshima Y, Morishima T. 5-S-cysteinyldopa in urine and tumors. J Dermatol 1992; 19: 806–808.
21. Gasparini G, Bonoldi E, Viale G, Verderio P, Boracchi P, Panizzoni GA, et al. Prognostic and predictive value of tumour angiogenesis in ovarian carcinomas. Int J Cancer 1996; 69: 205–211.
22. Anderson D, Najafzadeh M, Gopalan R, Ghaderi N, Scally AJ, Britland ST, et al. Sensitivity and specificity of the empirical lymphocyte genome sensitivity (LGS) assay: implications for improving cancer diagnostics. FASEB J 2014; 28: 4563–4570.
23. Duncan LM, Berwick M, Bruijn BH, Byers R, Mihm MC, Barnhill RL. Histopathologic recognition and grading of dysplastic melanocytic nevi: an interobserver agreement study. J Invest Dermatol 1993; 100: 318S–321S.
24. Goldstein AM, Tucker MA. Dysplastic nevi and melanoma. Cancer Epidemiol Biomarkers Prev 2013; 22: 528–532.
25. Ito S, Fujita K. Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. Anal Biochem 1985; 144: 527–536.
26. Salopek TG, Yamada K, Ito S, Jimbow K. Dysplastic melanocytic nevi contain high levels of pheomelain: quantitative comparison of pheomelanin/eumelanin levels between normal skin, common nevi, and dysplastic nevi. Pig Cell Res 1991; 4: 172–179.
27. Arakawa H, Hara H, Suzuki H, Wakamatsu K, Ito S. Micro-analyses of eumelanin and pheomelanin in tissues of various melanocytic lesions. Jpn J Dermatol 2004; 114: 1682.
28. Matthews TE, Piletic IR, Selim MA, Simpson MJ, Warren WS. Pump-prove imaging differentiates melanoma from melanocytic nevi. Sci Transl Med 23 February 2011 3: 71ra15. DOI: 10.1126/scitranslmed.3001604.