Genome profiling of melanocytic tumors using multiplex ligation-dependent probe amplification (MLPA): Its usefulness as an adjunctive diagnostic tool for melanocytic tumors

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SUMMARY

Background: The histopathology of melanocytic tumors sometimes presents diagnostic problems. Applicable parameters other than routine pathology are needed.

Objective: We assessed the feasibility of multiplex ligation-dependent probe amplification (MLPA), a novel PCR-based genome profiling method, in the classification of melanocytic tumors.

Method: We extracted DNA from paraffin-embedded tissue sections of 24 primary melanomas, 14 Spitz nevi and 17 common melanocytic nevi. We analyzed the copy number gains or losses of a total of 76 genes spanning almost all chromosome arms using commercially available MLPA kits.

Results: Although four melanocytic nevi and three Spitz nevi did not yield sufficient DNA for reliable analysis due to small tumor size, the MLPA analysis was feasible and applicable to the remaining 88% of samples. We found multiple genetic aberrations in primary melanomas. The total number of aberrations in each tumor ranged from 1 to...

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1. Introduction

The gold standard for the classification of cutaneous melanocytic tumors is histopathological diagnosis. Although it is accurate in the diagnosis of most of the lesions, there remain cases in which histopathological analyses do not permit unequivocal diagnoses [1]. Several studies have shown that there is a considerable disagreement, even among the expert pathologists, when faced with actual histological specimens of such cases [2–6]. Misdiagnosis or incorrect interpretation of melanocytic lesions can result in unnecessary psychological stress to the patients, under-treatment or over-treatment, and improper follow-up. Thus, further diagnostically applicable parameters other than routine pathology are needed. Although several works have explored the possible contribution of ploidy analysis [7], morphometry [8,9] and immunohistochemistry [10,11], none of them are uniquely or specifically diagnostic, or have gained acceptance in routine practice.

Recent works using comparative genomic hybridization (CGH) by Bastian et al. [12–14] have shown that the pattern of genomic aberrations differs significantly between melanomas and nevi. The vast majority of primary melanomas have multiple chromosomal aberrations, whereas melanocytic nevi and blue nevi do not have any [12]. Importantly, the genetic make-up of Spitz nevi that frequently cause diagnostic problems is also different from melanomas. The majority of Spitz nevi have normal karyotypes, whereas increased copies of chromosome 11p, which does not occur in melanoma, are found in a minority of Spitz nevi [13,14]. Thus, the measurement of chromosomal aberrations using CGH could be a powerful diagnostic tool in the classification of melanocytic tumors [15,16]. However, CGH is difficult to use in routine practice, because it is time consuming and laborious. Another drawback to CGH is that, although it can be applied to routinely fixed tissues, a relatively large amount of tissue is needed to obtain sufficient DNA (at least 500 ng for a single experiment) [17].

2. Materials and methods

2.1. Tissues

Paraffin-embedded tissues of 24 primary cutaneous melanomas, 17 melanocytic nevi and 14 Spitz nevi were retrieved from the archives of the Department of Dermatology, Shinshu University Hospital, Matsumoto, Japan, Department of Dermatology, Kanazawa University Hospital, Kanazawa, Japan, and the Sapporo Institute for Dermatopathology, Sapporo, Japan. The primary melanomas included 16 acral lentiginous melanomas, 5 superficial spreading melanomas, 2 lentigo maligna melanomas and 1 mucosal melanoma. The thickness of the primary tumors ranged from 1.5 to 13 mm, median 4.0 mm. To obtain normal control DNA, paraffin-embedded normal lymph node samples were obtained from six melanoma patients (three males and three females) who underwent lymph node dissection. The study was approved by the medical ethical committee of the Shinshu University School of Medicine.

2.2. DNA extraction

DNA was extracted from 10-μm paraffin-embedded tissue sections. One to five tissue sections were...
collected in a microtube containing 200 µl of P-buffer (50 mM Tris-HCl pH 8.5, 100 mM NaCl, 1 mM EDTA, 0.5% Tween-20, 0.5% NP40, 20 mM DTT) and heated at 90 °C for 15 min. After being cooled to 60 °C, 2 µl of 20 mg/ml proteinase K (Promega, Madison, WI) was added to the tube, and the tissues were incubated at 60 °C for 16 h. In some samples, incubation with proteinase K was extended up to 72 h at 56 °C with an addition of fresh proteinase K every 24 h. After the heat inactivation of proteinase K at 95 °C for 5 min, the tubes were centrifuged, and 150 µl of supernatant was precipitated with ethanol. Pellets were dissolved in 30–50 µl of TE buffer. DNA samples of normal lymph nodes from three males and three females were, respectively, admixed and used as sex-matched controls.

2.3. MLPA reaction

We performed MLPA analyses using SALSA P005 and P006 Chromosomal Aberration MLPA Kits (MRC-Holland, Amsterdam, Netherlands) following the manufacturer’s protocol. The P005 and P006, respectively, contained 41 probes which target a total of 76 genes (details available at http://www.mlpa.com). Briefly, 5 µl of DNA samples were heated at 98 °C for 5 min. Normal control DNA of males and females were always included in the same reaction. After the addition of the probe mix, samples were heated for 1 min at 95 °C and then incubated for 16 h at 60 °C. Ligation of the annealed oligonucleotide probes was performed for 15 min at 54 °C in buffer containing Ligase-65 enzyme. After inactivating the ligase enzyme by heating at 98 °C for 5 min, multiplex PCR was carried out using FAM-labeled primers, dNTPs and SALSA polymerase. PCR was performed for 33 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C. All the reactions were carried out in a thermal cycler equipped with a heat lid (iCycler, Bio-Rad Laboratories, Hercules, CA, USA). PCR products (1.5 µl each) were heat denatured and analyzed on a Gene Scan ABI-310 capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA).

![Female control](image1)

![Female melanoma P3](image2)

**Fig. 1** Representative examples of the MLPA electrophoresis profile of a control and a melanoma DNA using a SALSA P005 Chromosome Aberration Kit. Since both samples are female DNA, there are no peaks of the UTY gene on chromosome Yq11.1 (arrow). Low input DNA quality control peaks (white arrowheads) and a high ligation-dependent control peak (black arrowheads) indicate a good experiment with sufficient amounts of DNA. The melanoma sample shows multiple copy number gains (green) and losses (red) of genes indicated in the figure. Copy number loss includes the CDKN2A gene.
2.4. MLPA data analysis

If the amount of sample DNA is insufficient for reliable analysis, the amplification products of the ligation-independent quality control fragments will be prominent compared to the ligation-dependent control peak (i.e., ligation-independent DNA control peak values above 20% of mean peak height or DNA ligation control peak value less than 80% of mean peak height) (Fig. 1). These samples were excluded from the analyses, and only the samples containing sufficient DNA were analyzed. The Gene Scan data of sizes and peak areas of multiplex PCR products were exported to an Excel file. Non specific amplification products and primer dimer peaks were removed due to their low peak and/or short length. All the expected MLPA products were normalized by dividing each peak area by the combined peak area of all peaks in that lane (relative peak area). The relative copy number for each probe was expressed as a ratio of the relative peak area for each locus of the sample to that of relative peak area of the normal sex-matched control. Similar calculations were also performed for peak heights. There were no significant differences in the outcome of normalized areas and heights in most of the samples. A difference was considered significant if the both ratios calculated from areas and heights were less than 0.7 (loss) or higher than 1.3 (gain) [19,20].

2.5. Receiver operator characteristic (ROC) curve analysis

To assess the validity of the MLPA analysis in a diagnostic setting, data sets of the total numbers of losses and gains in primary melanomas and Spitz/melanocytic nevi were analyzed using SPSS 11.5J software. Threshold number of genomic aberrations corresponding to 98% specificity for melanoma were obtained by the linear regression of the two data...
points whose 1-Specificity values were less than 0.25, and solved for the number of aberrations corresponding to a 1-Specificity of 0.02 [21].

3. Results

All 24 melanoma samples showed low peaks of ligation-independent quality control fragments and high ligation-dependent control peaks, indicating that sufficient amounts of DNA were obtained for reliable analyses (Fig. 1). However, because of the small tissue size, four melanocytic nevi and three Spitz nevi did not yield sufficient DNA, and thus, were excluded from the analyses. These nevi with nevus cells proliferating only in the epidermis and papillary dermis. Fig. 2 shows the copy number changes of all the analyzed tumors in a total of 76 genes spanning almost all chromosome arms that were included in the P005 and P006 probe mixes.

Primary melanomas showed multiple copy number losses and gains. The total number of aberrations in each tumor ranged from 1 to 32, and the average number of copy number changes was 12.04. All but two melanomas (P40 and P93) showed aberrations at more than three genetic loci. P40 was an 83-year-old female with acral lentiginous melanoma 3 mm in thickness, who developed multiple metastases and died 6 months after surgery. P93 was a 41-year-old male with 4 mm thick acral lentiginous melanoma with positive sentinel node. He is free from disease 1 year after inguinal lymph node dissection. Although copy number gains or losses were observed for most of the genes, 17 of the 24 tumors (70.8%) showed the loss of either the CDKN2A or CDKN2B gene, which mapped to chromosome 9p21, the region most frequently deleted in primary melanomas [22,23].

By contrast, Spitz nevi and melanocytic nevi showed few genetic changes. The average number of copy number changes in Spitz and melanocytic nevi was 1.04. All the Spitz nevi showed one or two copy number changes. Distribution of the altered genes in Spitz nevi was random. Although it was reported that about 10% of Spitz nevi had copy number increases of the HRAS gene (11p15.5) [13,14], none of our Spitz nevus cases showed aberrations at this locus. Six of 13 melanocytic nevi had one aberration, and one nevus had aberrations at two loci.

The results of ROC curve analysis are shown in Table 1. The area under ROC curve was 0.973 which meant high accuracy of MLPA analysis in the diagnosis of melanoma. The threshold value of copy number aberrations corresponding to a specificity level of 98% was 2.42. The sensitivity of MLPA diagnosis using this threshold value was 92.5%.

4. Discussion

Although a clinical test that could enhance established diagnostic procedures would be of significant clinical benefit in the management of patients with melanocytic tumors, currently there is no method of definitively resolving the ambiguities of histopathological diagnosis. Such a test should be applicable to routine fixed tissue, because diagnostic problems typically arise after the specimen has already been fixed and processed. The advantage of MLPA is that the procedure is simple, multiple samples can be analyzed in a single reaction, and it can be performed using only 3–5 ten-micron paraffin sections [18]. Very recently, van Dijk et al. [17] critically evaluated the MLPA technique for the detection of DNA copy number changes in DNA isolated from formalin-fixed paraffin-embedded tissue that were previously characterized by CGH. They found that 86% of the loci tested by MLPA were concordant with CGH results, and concluded that MLAP is a reliable and efficient method to evaluate DNA copy number changes in formalin-fixed paraffin-embedded tissue. In this study, we examined a series of primary cutaneous melanomas, Spitz nevi and common melanocytic nevi by using the same commercially available MLPA kits. The two MLPA probe sets, P005 and P006, contained probes that target a total of 76 cancer-related genes spanning almost all chromosome arms. Although sufficient amounts of DNA for reliable analysis were not obtained in 7 (12%) tumors (all of them were small compound nevi and Spitz nevi), the method was feasible and applicable to most of the cases.

The overall results of copy number changes in melanocytic tumors were similar to those reported previously using CGH [12,23]. Most of the primary
melanomas showed copy number aberrations of multiple genes, whereas Spitz nevi and common melanocytic nevi showed few aberrations. The ROC curve analysis revealed that a total number of genomic aberrations could be an accurate diagnostic marker discriminating between melanoma and benign nevus. The threshold value of 2.42 copy number aberrations was 98% specificity and 92.5% sensitivity for melanoma. Nevertheless, 2 (8.3%) of the 24 melanomas had copy number changes at only one or two genetic loci, which were below the threshold value of 2.42. Both cases had lymph node metastases, one died of multiple visceral metastases. Bastian et al. [12], also reported that 7 (3.8%) of 133 primary melanomas had no chromosomal aberrations detected with CGH. Thus, a minor proportion of melanomas seem to have few chromosomal aberrations, which is likely to limit the genomic analyses in the diagnostic use. It should also be mentioned that contamination of stromal cells may dilute the tumor DNA and may lead to false results of normal copy number in CGH as well as in MLPA analyses.

The previous study by Bastian et al. [13,14], showed that the majority of Spitz nevi had no chromosomal aberrations, whereas the increased copies of chromosome 11p were found in a minority of cases. However, our MLPA analyses detected copy number changes at one or two genetic loci other than 11p in all the Spitz nevi examined. This may not be surprising because the resolution of CGH is poor (10–20 Mb), whereas MLPA can detect copy number changes of one particular gene. In keeping with our MLPA results, loss of heterozygosity (LOH) or allelic imbalance (AI) studies using PCR-based microsatellite analysis also revealed a few LOH or AI on chromosomes 5, 8 and 9 in a minority of Spitz nevi [22,24]. Thus, Spitz nevi may actually have copy number gains and losses of several genes. However, this needs to be confirmed by other techniques, such as fluorescent in situ hybridization (FISH) or high resolution array-based CGH, because both MLPA and LOH/AI analyses rely on PCR, which can generate artifacts when DNA templates extracted from paraffin-embedded tissues are used [25,26]. van Dijk et al. [17] actually pointed out that MLPA may occasionally yield false–positive results as normal samples show gains or losses in 7.3% of the probes analyzed.

Chromosome gains or losses in primary melanomas are not random, and several selected chromosome loci are preferentially affected. The most frequently detected aberration is the loss of chromosome 9p21 where CDKN2A and CDKN2B genes reside (Fig. 2) [22,23]. In line with these earlier findings, our MLPA analyses showed that 70.8% of primary melanomas had copy number losses of either the CDKN2A or CDKN2B gene. Because none of the Spitz nevi and common melanocytic nevi showed copy number losses of these two genes in our analyses, this may help to discriminate melanoma from benign nevus. Similarly, detection of copy number changes of other genes or chromosome loci that are frequently and specifically altered in melanoma would be helpful in differentiating melanoma from nevi. These may include genes such as PTEN (10q23.31) [27], CRSP3/DRIP130 (6q16.3-q23) [28], BRAF (7q34) [29], CCND1 (11q13) [30] as well as yet unidentified genes on frequently altered chromosome regions including 1q, 5p, 6p, 8p, 8q and 11q [23]. Unfortunately, however, currently available MLPA probes do not contain most of these genes and chromosome regions. Future efforts should be directed to the selection of appropriate genes discriminating melanoma and nevi and to the construction of MLPA probe sets covering such genes. The MLPA analysis using such customized probe sets would be a simple and easy molecular test in the classification of melanocytic tumors.

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