Direct Visualization of the Clonal Progression of Primary Cutaneous Melanoma: Application of Tissue Microdissection and Comparative Genomic Hybridization

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Abstract

Human cutaneous malignant melanoma progresses through a series of well defined clinical and histopathological stages. It has been assumed that the neoplastic progression of this disease advances from a common acquired nevus or dysplastic nevus through the primary radial growth phase (RGP), primary vertical growth phase (VGP), and finally to distant metastasis. However, it has never been directly shown that VGP is clonally derived from RGP. Furthermore, it has not been possible previously to conduct a detailed genetic analysis on pure tumor cells from archival material because the lesions are a heterogenous mixture of normal and neoplastic cells, and the entire specimen must be excised and fixed for clinical diagnosis. This report describes a new approach designed to identify DNA copy number changes in tumor cells from a series of progressive primary stages of cutaneous melanoma archival biopsies. Under direct high-power visualization, cells are procured with a sterile needle from highly specific areas of the tissue section. DNA is extracted from microdissected cells (normal, RGP, and VGP), PCR amplified, fluorescently labeled, and examined by comparative genomic hybridization to determine DNA copy number changes. Data obtained from three representative cases suggest a clonal derivation of VGP cells from RGP. This approach could be useful in identifying the sequence of genetic changes in progressive cutaneous melanoma stages.

Introduction

Superficial spreading melanoma is the most common form of human cutaneous melanoma and accounts for approximately 80% of all cases (1). Clinical and histopathological studies suggest that the progression of superficial spreading melanoma initiates as a putative precursor stage, common acquired nevus or dysplastic nevus, and advances through a primary RGP, 2 primary VGP, and finally to distant metastasis (1–3). Common acquired nevi are well circumscribed, flat, or raised hyperpigmented lesions generally regarded as a precursor stage, common acquired nevus or dysplastic nevus, and the neoplastic progression of this disease advances from a common acquired nevus or dysplastic nevus through the primary radial growth phase (RGP), primary vertical growth phase (VGP), and finally to distant metastasis. Therefore, cyto genetic (6–9) and molecular (3, 4, 10, 11) studies of tumor biopsies have been primarily confined to metastatic melanoma and established cell lines. These data indicate that chromosomes 1, 6, 7, 9, 10, and 11 are frequently altered in advanced melanoma. Due to the focus on the advanced melanoma cell lines, it is difficult to distinguish primary and secondary chromosomal alterations. Correlating the appearance of chromosomal alterations with the progressive stages of melanoma is an important first step in discerning primary from secondary genetic alterations.

This report describes a new approach that utilizes tissue microdissection (12, 13) to procure highly purified samples of melanoma tumor cells for subsequent analysis of DNA copy number changes by CGH (14, 15). Formalin-fixed, paraffin-embedded tissue sections with concomitant RGP and VGP were selected for study. The DNA was extracted from the cells, PCR amplified, fluorescently labeled, and analyzed by CGH. Three cases are reported illustrating complex numerical genetic changes in primary RGP and VGP stages, which suggest that the derivation of RGP and VGP cells are from the same clonal precursor population. The approach and data detailed in this report should help to identify potentially important genetic loci altered in distinct progressive stages of this cancer.

Materials and Methods

Tumor Samples. Formalin-fixed, paraffin-embedded tissue sections of primary human cutaneous malignant melanoma were obtained from the Southwest Oncology Group and the Eastern Cooperative Oncology Group. Data from three cases containing RGP and VGP in the same tissue section are reported in this article. The clinical and histological information of each case are summarized in Table 1.

Tissue Microdissection. RGP and VGP were carefully microdissected from the histological tissue sections as described previously (12). Briefly, hematoxylin and eosin-stained tissue sections were used to identify and locate desired groups of cells. The cells of interest were microdissected from a single 5-μm, deparaffinized, eosin-stained adjacent section (Fig. 1, A and B) with a modified 26-gauge sterile hypodermic needle. The cells were procured by electrostatic adhesion. Between 25 and 100 cells from histologically normal primary RGP and primary VGP tissues from each sample were collected separately in sterile tubes containing 20 μl of a DNA extraction buffer [50 μg/ml proteinase K-10 mM Tris-HCl (pH 8.0)] and incubated at 55°C over-
The mixtures were boiled for 20 min to inactivate the proteinase K, and 5 μl were used as a template to PCR amplify the genomic DNA.

**PCR Amplification.** The amplification of the extracted genomic DNA was performed with a degenerate universal primer as described previously (16). The following changes were made to the protocol. The initial PCR amplification step was carried out in a 10-μl reaction volume for 8–10 cycles, using 1 μM universal primer. The second amplification step was done in a 100-μl reaction volume for 35 cycles. The reaction mixture consisted of all the PCR products (10 μl) from the initial amplification step, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 μM of each dNTP, 1 μM universal primers, and 0.05 unit of Taq DNA polymerase. A 16 μl sample of the PCR products was loaded on a 1% agarose ethidium bromide gel to visualize the efficiency of the amplification (Fig. 2A).

**DNA Labeling.** The amplified genomic tumor DNA (test DNA) was labeled with FITC-conjugated dUTP by PCR. The reaction solution was comprised of 5 μl of the amplified genomic tumor DNA; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl₂; 0.001% gelatin; 0.2 μM each of dATP, dCTP, and dGTP; 0.05 mM dTTP; 0.12 mM fluorescein-12-dUTP (DuPont, Boston, MA); 4 μM universal primer; and 0.1 unit of Taq DNA polymerase. The PCR reaction was carried out in a 25-μl volume under the following conditions. After an initial 5-min denaturation at 95°C, the reaction underwent 25 cycles of 1 min at 95°C, 1 min at 56°C, and a final 10-min extension at 72°C. The PCR products were purified from unincorporated nucleotides with Bio-Spin 6 chromatography columns (Bio-Rad Laboratories, Hercules, CA), and 5 μl were loaded on a 1% agarose, ethidium bromide gel to visualize the additional amplification (Fig. 2B). The PCR-labeling efficiency (percentage of the FITC-dUTP incorporation) and DNA concentration were determined by a UV spectrophotometer (Beckman DU 640). Approximately 3% of FITC-dUTP incorporation (incorporated FITC-dUTP/total nucleotides) and 100 ng/μl of DNA were consistently obtained.

The normal reference DNA was extracted from known normal male (GM01247B) and female (GM10959) lymphoblastoid cell lines. The DNA was labeled with Texas red 5-dUTP (DuPont) by nick translation.

**Comparative Genomic Hybridization.** The CGH analysis was performed according to Kallioniemi et al. (15) with modifications detailed below. Briefly, the normal metaphase spreads were prepared from normal peripheral blood lymphocytes and aged for at least 2 weeks at room temperature (17). The slides were denatured in 70% formamide/2× SSC (1× SSC = 0.15M NaCl and 0.015M sodium citrate) at 68°C, dehydrated in a series of ethanol washes (70, 85, and 100%), followed by treatment with 0.1 μg/ml proteinase K (20 mM Tris-HCl [pH 7.5]-2 mM CaCl₂) for 8 min, and dehydrated again before hybridization. Approximately 400 ng of Texas red-labeled normal reference DNA and FITC-labeled test DNA, along with 40 μg of Cot-1 DNA, were hybridized to normal metaphase spreads. After 3 days of hybridization, the slides were washed as described by Pinkel et al. (18), counterstained with 0.1 μg/ml DAPI, and examined with a Zeiss Axiophot microscope equipped with appropriate fluorescence filters and a CCD camera interfaced with a computer. Three images of each metaphase were captured using filter wheel-mounted, single band excitation DAPI, FITC, and Texas red filters and a cube-mounted triple band pass dichroic and emission filter (Chroma Technology Corp., Brattleboro, VT). The image and profile analyses were carried out using the image analysis application, SinApps (Resource for Molecular Cyto- genetics, Lawrence Berkeley Laboratory, University of California San Francisco, Berkeley, CA), based on the SCIL-Image program (TPD/TNO, Delft, the Netherlands) (19). At least six metaphases were analyzed to generate fluorescence ratio profiles in each case. Interpretation of the profiles was performed according to the guidelines described by Kallioniemi et al. (15).

### Results

Cells from regions of RGP and VGP were selectively microdissected from archival primary melanoma tissue sections obtained from three patients (Fig. 1, A and B, Table 1). Fig. 1A shows a representative eosin-stained tissue section used to procure RGP and VGP cells. The nest of RGP cells surrounded by normal squamous epithelial cells in the epidermal layer is microdissected without contamination of normal cells (Fig. 1B). The VGP has expanded throughout the dermal layer of the skin, providing a large region for microdissecting a highly purified sample of VGP cells (Fig. 1B).

The microdissected cells were treated with proteinase K to release the genomic DNA, which was PCR amplified and FITC labeled with the UN-1 universal primer. The results of the DNA amplification were visualized on ethidium bromide-stained agarose gels (Fig. 2). The size range of the amplified DNA isolated from tissue sections was consistently smaller (150–1000 bp) than the product from genomic DNA isolated from cell lines (200 to over 2000 bp), suggesting fragmentation of tissue sectioned DNA (Fig. 2A). A sample of the amplified DNA was PCR labeled with a FITC-dUTP (Fig. 2B). The size range of the labeled DNA is similar to that of the initially amplified DNA (Fig. 2A).

Analysis of the entire tumor genome for gains and losses of DNA copy number was carried out using CGH. As a control for the fidelity of the procedure, normal female DNA isolated from an established lymphocytic cell line (GM10959) was amplified and FITC-dUTP labeled as described above. This FITC-labeled test DNA was hybridized to normal metaphase spreads along with Texas red-labeled normal female reference DNA and Cot-1-blocking DNA. The analysis revealed no numerical deviation from the normal diploid genetic complement, which was expected. However, chromosome 19p showed a fluorescence ratio below the normal ratio of 1.0, suggesting a false positive interpretation of a deletion (15). Chromosomes X and Y also gave false positive CGH profiles. Therefore, the profiles from chromosomes 19, X, and Y were excluded from the CGH analysis. These results indicate that the PCR amplification and labeling of the DNA was sufficiently representative for CGH analysis.

The results of the CGH analysis of three reported cases are summarized in Table 2. The results illustrate numerical alterations present in both VGP and RGP stages. The CGH profile of case 1690-640 revealed loss of chromosome 1pter–p33 in both VGP and RGP stages, and chromosomes 16 and 22 were lost in the RGP and VGP stages of 1690-641. In addition, DNA copy number changes were seen in the VGP that were not observed in the corresponding RGP. Gain of genetic loci on chromosomes 4, 11, and 13 was observed in VGP that were not observed in the corresponding RGP. Gain of genetic loci on chromosomes 4, 11, and 13 was observed in the VGP cells of 1690-640 and 1690-641, but no numerical changes of these chromosomes were observed in the RGP cells. Also, gain of chromosomes 6p, 6q12–q24, and 7 was identified in the VGP cells of 1690-550, 1690-641, and 1690-640, respectively. An example of a numerical genetic change in VGP cells of case 1690-641 is illustrated in Fig. 1C. The CGH profile and a representative digitized image of the hybridization to chromosome 7q are presented, using DNA isolated from RGP and VGP cells. Gain of genetic material on the terminal portion of 7q was observed with the CGH analysis of DNA from VGP cells but not from RGP cells. The high profile ratio and intense green hybridization signals suggest genomic amplification of this region.

Comparing the DAPI- and FITC-captured digital images localized the highest intensity amplification signal to 7q32-34 (Fig. 1C). The high ratio observed in the CGH profile from RGP cells of chromosome 7 was caused by insufficient blocking of the centromeric regions by Cot-1 DNA (Fig. 1C). This was verified by visually examining the captured digital images of each hybridization.
Discussion

This article focuses on a new approach designed to analyze the genome of tumor cells isolated from progressive stages of human superficial spreading melanoma. Two recently developed techniques, tissue microdissection and CGH, were combined to isolate specific tumor cells from tissue sections and analyze the genomic DNA. It is difficult to study primary melanoma samples using standard techniques because they are routinely fixed and sectioned for clinical diagnoses. The procedure used here takes advantage of the availability of formalin-fixed, paraffin-embedded melanoma tissue sections to examine genomic tumor DNA.

It is difficult to obtain pure homogeneous samples of tumor cells from a melanoma tissue section because the cells are in close proximity to normal cells and other tumor cells. Tissue microdissection overcomes this difficulty by selectively dissecting RGP and VGP cells from the archival tissue without contamination from surrounding normal cells (Fig. 1, A and B). PCR is then used to amplify and fluorescently label the DNA isolated from the cells (Fig. 2). The quality of the DNA obtained by this procedure appears sufficient for CGH analysis, avoiding the necessity of using multiple or thick (50–100 μm) tissue sections to obtain high molecular weight DNA for molecular genetic analysis (20–22).

CGH analysis was performed on samples of microdissected normal, RGP, and VGP cells procured from three cases of primary malignant melanoma. Genetic material from chromosome 17 was lost in both RGP and VGP of cases 1690-640 and 1690-641 (Table 2). These data are consistent with the molecular studies reporting loss of chromosome 17 loci in metastatic and primary melanoma samples (3, 4). The loss of 1pter–p33 observed in cases 1690-640 and 1690-641 is also consistent with previously published studies illustrating chromosome 1p36 being frequently lost in malignant melanoma cell lines (4, 10). Chromosomes 16 and 22 were both lost in the RGP and VGP cells of case 1690-641, although it has not been reported previously that these chromosomes were frequently altered in melanoma. Taken together, the results imply that the VGP cells were derived from the RGP cells in these cancers.

The CGH profiles showed a disparity between RGP and VGP cells. An example of this is the gain of chromosome 6p only in the VGP of case 1690-550 (Table 2). This is consistent with several cytogenetic reports documenting the presence of isochromosome 6p in metastatic

Fig. 1. Illustration of microdissection-CGH. A, an eosin-stained tissue section of a melanoma biopsy with a nest of RGP cells surrounded by normal squamous epithelial cells (upper box) and a large VGP region in the dermal layer (lower box). B, microdissected RGP and VGP cells. (×100). C, representative digitized images and profiles of CGH to chromosome 7 with isolated DNA from microdissected RGP and VGP cells (arrows) of case 1690-641. CGH profile is displayed below each image. A ratio of 1.0 represents neither gain nor loss of genetic material from microdissected cells. The dotted lines above and below the baseline represent ratios of 1.5 and 0.5, respectively. Idiogram shows the amplified region (green) 7q32-34 identified in VGP cells.
melanoma (6). Additionally, the CGH profiles of the VGP cells in two of three cases (1690-640 and 1690-641) illustrate a whole and partial gain of chromosome 7, respectively (Table 2, Fig. 1C). Observations of three cases (1690-640 and 1690-641) illustrate a whole and partial loss of genetic material: NA, no analyzable hybridization obtained. NA, neither a gain nor a loss of genetic material; NA, no analyzable hybridization obtained. Bold type indicates same genetic alteration present in the RGP and VGP stages.

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**References**


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