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INCREASED CHROMOSOME 20 COPY NUMBER DETECTED BY FLUORESCENT IN SITU HYBRIDIZATION (FISH) IN MALIGNANT MELANOMA

by
James Harold Barks

A Thesis Submitted to the Faculty of the
GRADUATE INTERDISCIPLINARY PROGRAM IN GENETICS
In Partial Fulfillment of the Requirements
For the Degree of
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STATEMENT BY AUTHOR

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DEDICATION

This work is dedicated to the memory of Mr. Floyd Thompson, whose expertise in the field of cancer cytogenetics and sense of humor will be sorely missed by myself and his colleagues in the coming years. I would also like to dedicate this thesis to the memory of Miss Stephanie Rodriguez, whose courage in the face of death was a model for me when things got rough. Lastly, I would like to dedicate this work to my family, whose moral and financial support were invaluable to me at this time and so many others.

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ABSTRACT

DNA amplification is an important mechanism of tumor progression that allows cancer cells to up-regulate expression of critical genes such as oncogenes. Recent studies using comparative genomic hybridization revealed increased DNA copies on chromosome 20q in seven melanoma cell lines and eight archival metastatic melanoma lesions. We performed FISH analysis of metaphase spreads in 13 melanoma cell lines and nine primary melanoma specimens using a variety of probes specific for chromosome 20. All 13 cell lines (100%) and 8/9 primary tumors (89%) showed extra copies of chromosome 20 relative to tumor ploidy. Additionally, 6/14 cell lines (43%) and 2/8 primary tumors (25%) showed translocated chromosome 20 material. Cytological evidence for gene amplification was found in one of the 13 cell lines with an add(20)(p13). These data suggest that over-representation of a gene(s) important for melanoma pathogenesis occurs on the chromosome 20 long arm.

INTRODUCTION

The Etiology and Biology of Melanoma

Malignant melanoma is a tumor that affects melanocytes, the pigment-producing cells located in the epidermis. The tumor is a relatively common neoplasm and essentially the only cutaneous malignancy that metastasizes and causes death. In 1992, an estimated 32,000 cases of malignant melanoma occurred in the United States, resulting in 6,700 deaths (1). In 1993, the annual rate of increase in malignant melanoma was estimated to be 7% per year, compared to a population increase of only 1.17% per year (2). This rate of increase is higher than any other malignancy rate in the United States, and is expected to raise the risk of a single individual developing malignant melanoma during their lifetime to 1 in 75 by the year 2001 (2). Median age at diagnosis is 40 years, so malignant melanoma affects young individuals during their most productive period in life.

The development of melanoma is multifactorial, with the predominant cause being the relationship between the genetically determined susceptibility of the host and the host's exposure to ultraviolet (UV) radiation. However, there are multiple other elements which affect melanoma growth as well: therefore, a brief discussion on the variety and relative importance of the factors believed to contribute to melanoma pathogenesis is relevant to this study.

The genetic composition of the human host can affect the incidence and occurrence of melanoma in a number of ways. For example, genetic factors as expressed in race and skin pigmentation type are among the most important for researchers to consider when evaluating worldwide incidence of melanoma (2). The process of skin pigmentation occurs when melanocytes transfer melanin granules organized into melanosomes to the surrounding keratinocytes; the phenotypic expression of skin color results almost entirely from the concentration of melanin in these two cell types (3). Melanin granules may exert a protective effect by absorbing UV radiation, thereby preventing damage to the DNA of the melanocytes and other epidermal cells. Consequently, the incidence of melanoma should be lowest among the darkest skinned or Negroid races, higher among the less dark Asians, and highest among Caucasians. In general, this trend has been found to be true: the mean incidence of melanoma among Caucasian populations is over three-fold greater than that in non-whites (4). Caucasians as a race have the highest rate of malignant melanomas, and it has been postulated that a Celtic or Nordic genetic background carries the greatest susceptibility (5). As the data regarding race suggest, the overall incidence of melanoma is

higher among Asians compared to Negroes (4). Although the existence of these racial trends has been well described, genetic predisposition to melanoma can also be defined by more specific factors than race or skin pigmentation. For example, studies of familial melanoma have shown genetic linkage to two loci, 1p36 (6) and 9p21 (7). In both familial studies, deletion of or loss of heterozygosity in the genes within these regions resulted in a higher likelihood for individuals in the affected families to develop malignant melanoma. Lastly, the incidence of malignant melanoma can be very high in patients with inherited DNA repair disorders such as xeroderma pigmentosum (4). In summary, there are a variety of different ways by which the genetic makeup of the host can affect the risk of developing melanoma.

In addition to genetic factors, a number of non-genetic elements exist that can increase melanoma risk, the most important being exposure to UV light. Several recent studies (8, 9, 10) have demonstrated increased risk of developing melanoma with UV exposure: specifically, these studies have shown that intermittent intense UV-B exposure in childhood and adolescence or use of UV tanning beds and lamps significantly increases the risk of melanoma development even after adjustment for other risk factors. Non-genetic factors other than UV light exposure can also significantly affect the incidence of melanoma, even within defined race and skin pigmentation groups. For example, different studies have shown that, in general, the risk of melanoma increases in peoples residing closer the equator (11) or belonging to the upper classes (12); while the former is due to the fact that solar radiation is more intense near the equator, the latter may be due to lifestyle choices such as tropical vacations and golf weekends which result in bursts of intense UV exposure. Additionally, differences in habits of dress between cultures and genders may account for the results seen in some studies. For instance, white females in general have a higher incidence of melanoma than white males due to a much higher occurrence of melanoma on the female leg (4). Lastly, multiple other factors such as hormones and diet continue to be evaluated as possible etiologies in the development of malignant melanoma (13).

Like many other cancer types, melanoma usually begins as a benign lesion and only becomes malignant after passing through a series of intermediate stages, a process called tumor progression. Tumor progression may be divided into indirect and direct pathways (14). Direct tumor progression is characterized by the appearance of cancer with metastatic competence in the first manifest lesion without evidence of precursor steps. In contrast, indirect tumor progression, which is the most common pathway, is a multistep process. Of

the four common biological forms of melanoma, three may give rise to metastases via indirect tumor progression: superficial spreading melanoma, lentigo malignant melanoma, and acral lentinginous melanoma. The fourth form, nodular melanoma, is a model for direct tumor progression (15). The common acquired melanocytic nevus, a focal proliferation of structurally normal cells, is often the first step in the progression of melanoma, followed by the dysplastic nevus with abnormal hyperplasia of melanocytes and atypia, and finally evolution to primary melanoma .

All primary melanomas except nodular melanoma (which constitues only 12% of melanomas) first present as relatively flat, multicolored lesions that expand slowly at their periphery. This type of expansion may be observed for months or years without the physician observing any qualitative change. This stage (Stage I) has been termed the radial growth phase (RGP) because of the lesions' tendency to enlarge along the radii of an imperfect circle. Histologically, the melanoma cells grow in all epidermal layers and are seen in the superficial dermis, where they are arrayed as individual cells and small cell clusters. There is no apparent focus of dermal growth and no dermal nest has an apparent growth advantage over other nests. The cells of the RGP are antigenically distinctive, euploid, and non-tumorigenic in nude mice (15, 16). RGP melanomas show clinical evidence of autonomous growth and, histologically, are invasive malignant melanomas with the capacity for intraepidermal growth and the properties required to traverse the dermal-epidermal basement membrane zone. However, in spite of the ability to invade, there is no evident competence for metastasis. In a study of 149 RGP cases followed longer than eight years, no metastases or deaths occurred due to disease (17).

The next step in melanoma tumor progression is called the vertical growth phase (VGP), a focal event occurring within the RGP that marks a shift in growth from the epidermis into the dermis. This step is manifested by the appearance of a population of cells in the dermis different from those of the RGP: these cells are disposed in a sphere-like cluster or a plaque that has a growth preference over the cells of the RGP. This phase of tumor growth is divided into two stages, Stage II or regional disease (defined as local, nodal, or intransit metastases) and Stage III or distant (metastatic) disease. Regardless of any other attributes, tumors which have entered the VGP result in death due to disease in 30% of cases (17).

In conclusion, clinical and histopathologic evidence suggests that the majority of malignant melanomas develop in a stepwise fashion (Figure 1) and are similar in form and biology to the metastases seen in other neoplasms (15).

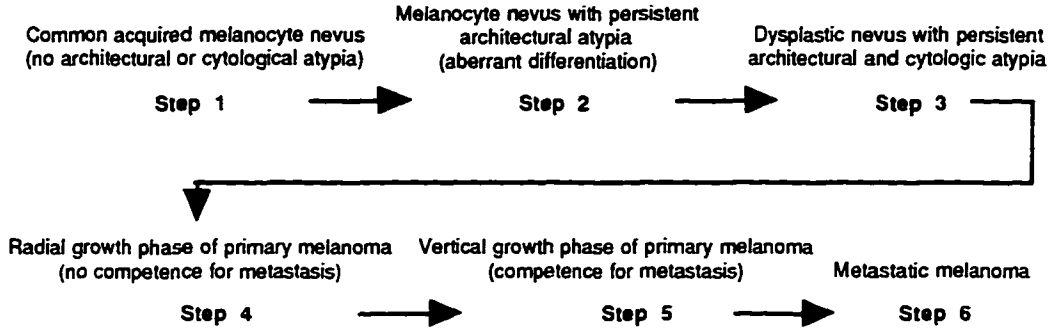


Figure 1 Tumor progression in human melanoma development.

The Cytogenetics of Human Neoplasms

One of the goals of modern cytogenetics is to define as thoroughly as possible the numeric and structural chromosome abnormalities associated with the different stages of tumor progression for a given cancer. This knowlege can then be correlated with patient information to provide a greater understanding of the clinical significance of cytogenetic observations. Toward this end, a great deal of literature on the topic of chromosomal changes in human malignancies currently exists, and reports of the cytogenetic evaluation of solid tumors have constituted an increasing proportion of that literature in the past few years. However, hematological malignancies, which constitute about 10% of human neoplasias, account for about 80% of the chromosome data currently available (18). This trend began with the discovery 30 years ago of the first consistent, specific chromosome abnormality associated with a human malignancy, the Philadelphia chromosome (Ph^1) found in the karyotypes of patients with chronic myeloid leukemia (CML)(19). Since then, advances in the understanding of chromosome abnormalities have continued to be made despite the presence of several technical stumbling blocks. These obstacles include (1) the development and refinement of cytogenetic techniques for obtaining and staining chromosomes; (2) the development of standards for describing and evaluating numerous, complex structural and numeric abnormalities in often highly heterogeneous populations (e.g., banding nomenclatures, clonal versus nonclonal abnormalities, and stemlines versus sidelines); (3) collection and analysis of clinical information such as histories, responses to treatments, and clinical outcomes; and (4) the development and application of molecular genetic techniques to clinical samples, both independent of and as a response to cytogenetic

observations. The first of these factors has limited the study of human solid tumors more severely than that of human hematological malignancies (18). Consequently, the clinical significance of consistent karyotype abnormalities in hematological malignancies was recognized earlier than in solid tumors. Because the technical approaches to analyzing acquired genetic abnormalities in hematologic malignancies continue to serve as models for the analysis of solid tumors, a brief discussion of the relationships between karyotypic abnormalities and clinical behaviors in hematologic cancers is relevant to this study.

As previously mentioned, the first karyotype anomaly described in human cancer was the Ph^1 chromosome. Improved chromosome banding techniques showed that the Ph^1 chromosome resulted from a balanced (meaning without chromosomal deletion), reciprocal translocation between chromosomes 9 and 22. Molecular genetic studies revealed that the balanced t(9;22) resulted in the translocation of the Abelson (ABL) gene from chromosome 9 band q34.1 (9q34.1) to the "breakpoint cluster region" (bcr) gene located in 22q11.21. This rearrangement created an abnormal reading frame containing a "fusion gene" made up of parts of both donor genes which encodes the bcr-abl protein, a constitutively active tyrosine kinase required in CML pathogenesis (20). Subsequent karyotypic analysis of other hematologic cancers demonstrated the existence of frequent, different, and recurring balanced translocations in leukemias and lymphomas (20). Like t(9;22), these translocations frequently create abnormal fusion genes or result in the increased transcription of genes not typically expressed in normal blood lymphocytes (19-21). Many of these abnormally expressed genes are transcription factors, but tyrosine kinases and other proteins can also be involved (21). The recurring karyotypic anomalies that signal the presence of these aberrant genes have been associated with specific clinical syndromes (i.e., t(15;17) and the resultant PML-RARa fusion gene are present in all acute promyelocytic leukemias) and show correlation with disease prognosis and therapeutic response (19-21).

The sequential analysis of blood cell tumor karyotypes revealed that chromosome abnormalities are retained during clonal and clinical progression, but additional karyotypic anomalies also frequently appear (22). More advanced states of disease (such as acute blast crisis in CML) and other forms of tumor progression are associated with cumulative numeric changes (i.e., +5, +16, -9), additional copies of the original or "primary" structural chromosome rearrangements, and/or the formation of new or "secondary" structural chromosome rearrangements. While structural rearrangements can form fusion genes and cause the abnormal expression of genes, numeric chromosome abnormalities can

unbalance the genome such that whole chromosomes or chromosomal segments may be over- or under-represented relative to normal diploid progenitor cells. These kinds of genomic perturbations may result in the over-representation of genes contributing to a cancerous phenotype or the deletion of non-mutated suppressor genes. In summary, it was the discoveries of these types of genetic lesions in hematologic cancers that inspired the search for similar chromosomal phenomena in solid tumors.

As mentioned previously, it was the creation and improvement of cytogenetic protocols for acquiring and banding chromosomes that most hampered efforts to characterize the karyotypic abnormalities of solid tumors. The technical problems associated with obtaining sufficient quantities of quality metaphases from solid tumors are due to the basic nature of the samples, the amount and type of processing that is required prior to chromosome analysis, and the biological characteristics of the tumor cells themselves. Stated more specifically, these obstacles include (1) the disaggregation of solid tissue samples into single cells or small cell clusters prior to chromosome harvesting; (2) low cell viability owing to necrosis arising from circumstances such as hypoxic conditions *in vivo* or lengthy transit times to the lab; (3) the contamination of tumor samples by normal cells from surrounding tissues or infiltrating inflammatory cells; and (4) the fact that most solid tumors, especially metastatic ones, are composed of highly heterogeneous cell populations by the time they present clinically (18).

The last of these obstacles has made it most difficult to correlate non random chromosomal abnormalities with clinical outcomes because the karyotypic anomalies usually found in solid tumors are quite complicated. Currently, there is a great deal of evidence (especially cytogenetic evidence) suggesting that solid tumors arise from a common progenitor stem cell that undergoes a malignant transformation and subsequently continues to evolve and diverge (22). This initial transformation step may be the result of a molecular mutation event (base substitution, insertion, deletion, etc.) and/or more broad genomic alterations such as chromosomal translocations or deletions. After the initial transformation process is completed, most solid tumors accumulate multiple additional chromosomal rearrangements and imbalances until the time they present clinically and are treated or removed. As a result, no two karyotypes from the same specimen may be exactly alike and perhaps 1 to 100 numerical and structural rearrangements may be described within each karyotype. This extensive genetic heterogeneity creates complex descriptions of **clonal mainline** (predominant); **stemline** (the clone with the simplest karyotype from which other subpopulations may be derived); and **sideline** (minor

subpopulations) abnormalities in solid tumors that are extremely unusual problems in hematological neoplasms (23). In addition to the difficulties encountered by cytogeneticists when characterizing numerical and structural anomalies, the range of chromosome counts may be quite wide (e.g., from hypodiploid to hyperpentaploid), and it is not uncommon to find more than one modal number. In many situations, a modal range may be found that includes a concentration of chromosome counts rather than a single number. Consequently, the results of solid tumor chromosome studies are typically complex and challenging to summarize. Despite these problems, it has been possible to describe certain chromosomal abnormalities that are specific for given tumor types and may represent primary changes important to early stages of transformation. Also, other nonrandom chromosomal abnormalities associated with varying clinical and molecular significance have been observed. Still, the difficulties of obtaining sufficient quantities and quality of complete karyotypes from solid tumors have resulted in the relatively low availability of information concerning the correlations between chromosomal abnormalities and clinical outcomes in solid tumors such as melanoma (18).

Melanoma Cytogenetics

The neoplastic system that affects epidermal melanocytes and leads to malignant melanoma is a valuable model for studying the sequential steps involved in tumor progression. The lesions of interest usually occur on the skin and are consequently accessible for early diagnosis and, when surgically removed, karyotypic analysis (24). As of 1995, approximately 200 complete malignant melanoma karyotypes derived almost exclusively from metastatic rather than primary tumors had been published (18, 24, 25). Unlike many other tumor types, malignant melanomas are relatively easy sources of chromosomes, essentially never normal (>99%)(26), and generally quite complex in their karyotypic alterations. Multiple structural and numeric chromosome abnormalities are often present simultaneously (23), while classic, specific gene amplification in the form of double minutes (DMs) or homogeneously staining regions (HSRs) is not as common as in other solid tumors. Although clonal abnormalities are present, the consistent, sequential accumulation of additional chromosome abnormalities has not been documented frequently during melanoma tumor progression. Recurring, consistent reciprocal translocations are not usually observed, but it is unclear whether this is due to the complexity of melanoma karyotypes or fundamental differences in the types of genetic transforming events that occur in hematologic and solid tumors (23). To date, the chromosomes most frequently

involved in both structural and numerical abnormalities in melanoma are 1, 6, 7, 9, 10, and 11 (23). The most common imbalances are loss of material from 1p, 6q (often because an i(6p) is formed) and 9p, gain of 6p and one copy of chromosome 7, and loss of one copy of chromosomes 9 and 10.

Gene Amplification In Tumorigenesis

The amplification of DNA sequences is known to be an important aspect of tumorigenesis, allowing cancer cells to up-regulate expression of critical genes such as oncogenes or genes conferring drug resistance (27). Gene amplification, which has been observed in solid tumors and a small percentage of hematologic malignancies but not in normal human tissues, involves the selective overproduction of a chromosomal segment composed of a particular gene and varying amounts of neighboring, or flanking, sequences. The region that is amplified and maintained in the tumor cell's genome is referred to as an amplicon and can vary in size. Cytologically, gene amplification is often signalled by the presence of DMs, HSRs, or abnormally banded regions (ABRs) of different chromosomes. DMs are small, paired bodies of chromatin (DNA and protein) of varying size that may occur in hundreds of copies per cell or in only a half-dozen. Their number may vary significantly from metaphase to metaphase within the same specimen because of uneven distribution to the daughter cells during cell division. HSRs are unusually extended regions of a chromosome that stain evenly and normally for the band where the HSR occurs (if on an identifiable chromosome). They can also vary in size and can sometimes increase the overall length of a chromosome manyfold. ABRs are similar to HSRs except that they may stain unevenly rather than homogeneously or may show a staining intensity that is slightly altered from that which is normal for the particular chromosome bands at which they are found.

How genes are amplified is not completely understood, but the process nearly always involves the movement of the amplicon's copies from its normal chromosomal location either to another chromosome where an HSR or ABR is produced after incorporation of these segments or to the extrachromosomal nuclear matrix, where the amplicon copies are found in the form of DMs. DMs and HSRs are not found together in the same metaphase spread unless they are derived from different amplicons (18). Generally, the presence of DMs, HSRs, or ABRs indicates more aggressive disease and poorer prognosis for the patient: on a clinical basis the specific overexpression of certain genes can, to an extent, reflect the magnitude of tumor aggressiveness and thus the

prognosis of a neoplasm (28). Therefore, the identification of increased gene copy number has direct clinical relevance to human malignancy.

Gene Amplification and Malignant Melanoma

Evidence of gene amplification has been found recently in a number of different neoplastic systems including breast cancers (29), ovarian cancers (30), and erythroleukemia (31). However, the extent of gene amplification in melanoma pathogenesis remains relatively poorly characterized.

To date, the most highly over-represented chromosome consistently found in melanoma is chromosome 7 (18): Koprowski et al. showed that the presence of extra chromosome 7 material was associated with an increased expression of the epidermal growth factor receptor gene located in 7p12-13 (32). However, some melanomas contain extra copies of 7q in the absence of 7p, suggesting that the melanoma-associated oncogene on this chromosome is located on 7q and not 7p. For example, a recent CGH study by Wiltshire et al. detected amplification of a region on 7q32-34 in the VGP, but not RGP, section of a metastatic cutaneous melanoma (33). In general, over-representation of chromosome 7 most frequently manifests as additional copies of the whole chromosome and is one of the very few instances in human neoplasia where extra copies of a chromosome have been directly correlated with a cellular change that may be contributing to the proliferative advantage of the neoplastic cells (24).

The short arm of chromosome 6 is also preferentially retained in melanocytic tumors, often as an isochromosome (24). When Sozzi et al. transfected a melanocyte cell line having a normal karyotype with a retrovirus containing H-*ras* and c-*myc* oncogenes, they induced all of the features of transformation and found that the only structural abnormality was an iso6p (34). However, the over-representation of chromosome 6p as a significant event in melanoma pathogenesis is not as compelling as the role of chromosome 7. This is largely because the most common abnormalities of chromosome 6 seen in melanoma are not the preferential amplification of 6p sequences but deletions in the long arm that result in the over-representation of 6p (24). In summary then, few chromosomes have been described as consistent sites of amplification in melanocytic neoplasms.

Molecular Cytogenetics and the Identification of Chromosome Segment Imbalances in Solid Tumors

The accurate identification of amplified DNA sequences in solid tumors using standard cytogenetic banding techniques has met with very limited success. The uniform staining of DMs and particularly HSRs makes it exceedingly difficult, if not impossible, to identify the origin of the involved amplicon if the amplicon exists extrachromosomally or if it has been translocated to a different chromosome. In the latter case, cryptic translocations of small segments of amplicons, especially to similarly staining regions of other chromosomes, can easily be missed by even the most experienced cytogeneticist. Furthermore, low-level (<five-fold) amplification of sequences may be present without cytologically recognizeable elements (35). Consequently, the identification of amplified sequences in solid tumors could only be done accurately using molecular genetic techniques. However, the development of molecular cytogenetic techniques, such as FISH, has revolutionized the accurate cytogenetic characterization of previously unidentifiable chromosome rearrangements by allowing cytogeneticists to directly visualize the origin, location, and number of DNA sequences or segments of particular interest.

The technique of DNA-DNA hybridization was initially introduced by Schildkraut, Marmur, and Doty (36). The first hybridization of radiolabeled DNA probes to cytological preparations was done in 1969 by Pardue and Gall (37) and was detected using autoradiography. This method remained in use for a number of years despite the demand for alternatives to radioisotopes to detect, localize, and isolate nucleic acid components. Aside from the risks associated with radioactivity, the low resolving power of autoradiography and long exposure times required made the aquisition of data tedious and time consuming. The first nucleotide analogs that could function as indicator "probes" when incorporated into polynucleotides were developed in 1981 by Langer et al. (38) when they synthesized biotin-conjugated dUTP. The coupling of this development with methods used for indirect immunofluorescence (39, 40, 41) created the progenitor of the FISH procedures currently in use. Multiple improvements upon the basic FISH procedure now allow cytogeneticists to analyze and karyotype metaphase spreads using single gene, region-specific, p or q arm-specific, and whole chromosome composite painting probes in addition to conventional banding techniques. Recently, the combination of two novel molecular cytogenetic techniques, chromosome microdissection and comparative genomic hybridization (CGH) (42), has made the search for amplified DNA sequences within tumor genomes even easier and more rapid.

Chromosome microdissection is a method for physically isolating a specific segment of a banded metaphase chromosome to obtain DNA for subsequent analysis (43-46). Recent applications have included the microdissection of whole chromosomes as well as individual chromosome bands to generate composite painting probes for FISH (45, 46). Another advantage of chromosome microdissection is the ability to dissect abnormal chromosomes (e.g., translocations, deletions, DMs, or HSRs) in addition to normal chromosomes. Once obtained, microdissected DNA can be amplified by PCR using a degenerate primer and subequently used as probes for FISH (35). These microdissected probes can then be used to define the chromosomal composition of DMs, HSRs and other chromosome anomalies or to serve as entry point clones for physical mapping studies (47). The disadvantages of microdissection are that the high cost of the equipment required for the procedure makes it inaccessible to most laboratories and, on a more technical level, it requires the presence of relatively high-quality chromosome preparations in order to be successful. In addition, it is also possible that gene sequences of interest may not be adequately represented in microdissected DNA because they are missed during the collection procedure (48).

The CGH technique allows the screening of genomic DNA to derive a "copy number karyotype" and consists of a FISH reaction in which tumor and normal DNA are simultaneously hybridized to normal metaphase chromosomes (42, 50, 51). This strategy can be used to determine the presence and location of amplified and deleted genomic regions of any tumor (42, 49-53). As initially described, nick translation is used to label tumor DNA with biotin and normal DNA with digoxygenin. Both probes are then simultaneously hybridized to normal lymphocyte metaphase chromosomes and visualized using fluorochromes (usually FITC and rhodamine) that render the tumor DNA green and the normal DNA red. The amount of each probe will vary along the length of a chromosome according to the copy number at which that region is represented in the tumor (35). Once a two-color FISH image is acquired, it is processed by software which calculates the intensities of each fluorochrome and subsequently generates a green/red ratio along the axis of each chromosome. Variations in this ratio correspond to deviations from the stemline copy number in the tumor: amplified tumor sequences will show a high ratio of green to red, while deleted regions will show a low ratio of green to red. Under optimal conditions, CGH is extremely sensitive to copy number changes (both deletions and amplifications) involving chromosomal segments with a minimum size of several Mb (35). The chief disadvantages of the CGH technique are that some chromosome regions (for

example, 1p36) can be difficult to resolve (54) and that the amplification and deletion of tumor sequences are described relative to diploid copy number and not the modal ploidy of the tumor being analyzed. Despite their respective disadvantages, the combination of microdissection and CGH has proven to be a very effective approach to the isolation and characterization of chromosome regions important in tumor pathogenesis.

The analysis of melanoma genomes by cytogenetics (CSRP) and/or CGH can also identify chromosomal regions important in oncogenesis and disease progression, though not as rapidly as microdissection and CGH. CSRP (chromosome segment representation profile) analysis relies on standard cytogenetic banding techniques and takes into account both gain and loss of normal chromosomes and the presence of structurally altered homologs, with the combined results producing a visual representation of the net gain or loss of chromosomes or chromosomal segments (55, see also Figure 2A). When used together, CSRP and CGH can complement each other in identifying novel altered chromosomal regions. For example, a pair of recent studies using these two techniques have demonstrated that chromosomes 1, 6, 7, 9, and 10 appear to harbor genes important in melanoma pathogenesis (26, 54), which is consistent with the melanoma literature accumulated to date. However, differences in DNA sequence copy number aberrations between CSRP and CGH were noted for chromosomes 15, 17, 19, 20, and 22, suggesting that these chromosomal regions may also contain genes important to melanoma development (54).

Statement of The Problem

The ultimate goal of genetic analysis of melanoma genomes is to identify the genes involved in melanoma development. We recently used comparative genomic hybridization (CGH) to screen melanoma cell lines and archival melanoma specimens for previously undetected chromosomal regions of loss or gain (54). The results from this study detected increased DNA copies on the the long arm of chromosome 20 in both the cell lines and the archival primary metastatic lesions (54, see also Figure 2C). This observation led us to the hypothesis that the long arm of chromosome 20 (20q) harbors a gene (or genes) that contributes to the malignant progression of melanoma. To test this hypothesis, the following specific aims were pursued: (1) To evaluate the nature of chromosome 20 abnormalities in melanoma in more detail, confirm both our karyotype and CGH findings, and resolve any discrepancies between techniques, FISH analysis of metaphase spreads from 13 melanoma cell lines and nine surgically resected primary melanoma specimens was performed using a chromosome 20 whole chromosome paint probe; (2) To determine if the long arm of chromosome 20 was involved in melanoma pathogenesis, FISH with a chromosome 20q microdissection probe (BT474) spanning the 20q11-20q13 region was performed; (3) To begin refining the region of most consistent amplification on 20q, FISH mapping with bacteriophage P1 clones specific for the 20q11 and 20q13 regions was conducted.

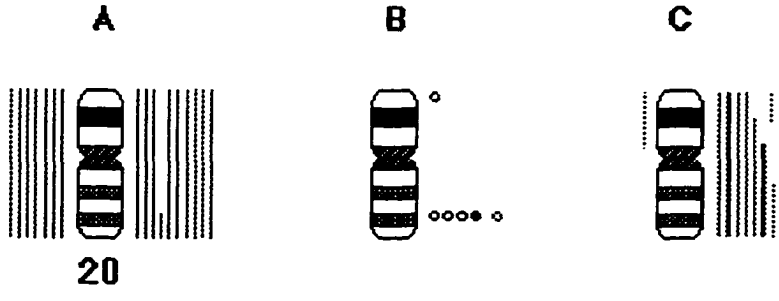


Figure 2 A) CSRP profile from a subset of 49 near-diploid melanomas showing gain of segments to the right and loss of segments to the left. B) Chromosome 20 breakpoints in 49 near-diploid malignant melanomas. C) Summary of CGH profiles from 7 melanoma cell lines showing gain of segments to the right and loss of segments to the left.

MATERIALS AND METHODS

Cells and Cultures: Melanoma cell lines were obtained from the Arizona Cancer Center Tissue Culture Core and cultured in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Biopsy specimens were mechanically and enzymatically disaggregated into single-cell suspensions and cultured in supplemented RPMI-1640 for an average of seven days prior to harvesting as previously described (26). Cells were grown in a 37°C incubator with a 5% CO_2 atmosphere. Upon reaching confluency in a 75 cm^2 flask (Costar, Cambridge, MA), cells were exposed to 0.05 µg/mL colcemid for 30-60 minutes before harvesting. After colcemid treatment, metaphases were prepared by removing the cells from the flask with 1X trypsin, centrifuging them at 1000 rpm for five minutes, and resuspending them in prewarmed (37°C) 0.075 M KCL hypotonic solution for 18-20 minutes. Cells were then fixed by washing three times for 20 minutes, 10 minutes, and 10 minutes each in 3:1 methanol/glacial acetic acid prior to slide preparation.

Slide preparation and karyotyping: Three to five drops of cell suspension were dropped onto glass slides (Fisher Scientific) precleaned with 95% ethanol. Slides were air dried overnight at room temperature and stained by the trypsin-Geimsa banding technique. Briefly, slides were soaked in room temperature Hank's buffered saline solution (HBSS, BioWhittaker) (pH 7.0) for 2-4 hours, denatured in HBSS/.08X trypsin (Intergen) for 2-3 minutes, rinsed twice in 70% EtOH, and Geimsa (Fisher Scientific) stained for 2-3 minutes. When possible, 25 metaphase spreads were analyzed and five photographed using a Genevision 121 Karyotyping System (Applied Imaging, Santa Clara, CA) to provide a detailed karyotype. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (1991 and 1995).

Fluorescent In Situ Hybridization: After G-banding, slides were destained by placing them in 100% EtOH for 10 minutes and rinsing them twice in 70% and 85% EtOH for one minute each. Slides were then washed in Carnoy's fixative for 10 minutes and allowed to air dry before washing in 3.7% formaldehyde/1X phosphate buffered saline (PBS) for 10 minutes. Slides were then rinsed twice in 1X PBS for five minutes each and dehydrated by successive washes in 70%, 85% and 100% EtOH for two minutes each. Slides were aged in 2X SSC (pH 7.0) for 30 minutes at 37°C and washed three times in 70

%, 85%, and 100% EtOH for two minutes each. Slides were denatured in 70% formamide/2X SSC (pH 7.0) for two minutes at 70°C and washed three times in ice cold 70%, 85%, and 100% EtOH for two minutes each and allowed to air dry. Ten µL/slide of digoxygenin-labeled COATASOME™ 20 Total Chromosome Probe (Oncor, Gaithersberg, MD) were prewarmed for five minutes at 37°C, denatured for 10 minutes at 70°C, and preannealed for two hours at 37°C before application to each slide. The probe mix was covered with a glass coverslip, sealed with rubber cement, and hybridized by placing the slide in a prewarmed, humidified chamber at 37° for 16-18 hours. Post-hybridization washing was done in 1X SSC (pH 7.0) for five minutes at 72°C without agitation and the slides were placed in 1X phosphate bufferred detergent (PBD) for five minutes before detection. Detection was done by applying 60µL of FITC-conjugated anti-digoxygenin to each slide and incubating under a plastic coverslip for five minutes at 37°C. Slides were rinsed in three changes of 1X PBD at RT for two minutes each prior to amplification. Digoxigenin signals were amplified by applying 60µL of rabbit anti-sheep antibody to each slide, incubating under a plastic coverslip for 15 minutes at 37°C, washing three times in 1X PBD for two minutes each with agitation, applying 60µL of FITC-conjugated anti-rabbit antibody to each slide, incubating the slide under a plastic coverslip for 15 minutes at 37°C, and washing the slide three times in 1X PBD for two minutes each with agitation. Cells were counterstained with 10 µL propidium iodide (final concentration 0.3 µg/mL in antifade)(Oncor) and examined using a Zeiss fluorescent microscope with a triple bandpass filter. When possible, 25 metaphase spreads were scored per sample, and photoimages were recorded on Kodak Royal Gold 1000 film using a 15 second exposure time.

The BT474 20q11-q13 region-specific microFISH probe and the 20q11 (3916 P1) and 20q13 (112G8 P1) band-specific P1 probes were obtained as generous gifts from the National Center for Human Genome Research. BT474 probe DNA was labeled as previously described (43) and 3916 P1 and 112G8 P1 DNAs were labeled using a Bioprime® DNA Labeling System (Life Technologies, Inc.) according to manufacturers protocol. Slide preparation and denaturation were done as described above. One µL of probe was mixed with one µL of CotI human DNA and eight µL of Hybrisol VI (Oncor), denatured for five minutes at 75°C, and prewarmed for 10 minutes at 37°C. The probe mix was then applied to a prewarmed (45°C) slide, sealed under a glass coverslip, and allowed to hybridize 16-18 hours. After hybridization, the slides were post-washed three times in 50% formamide/2X SSC (pH 7.0) for four minutes each, three times in 2X SSC (pH 7.0)

for four minutes each, and three times in 1X PBD for two minutes each. Detection, amplification, and imaging were conducted as described above.

RESULTS

Cytogenetic analysis

Karyotypes of the 13 melanoma cell lines used in this study are shown in Table 1. The majority of the cell lines (8/13) had a modal ploidy of ~3N. Six of 13 cell lines (UACC1529, UACC827, UACC1065, UACC1022, UACC1460, and UACC612) had karyotypes showing numeric abnormalities involving gain of chromosome 20, mainly in the form of +20. The UACC1227 and UACC502 cell lines were both -20, and UACC375 was -20, -20, but contained multiple copies of an add(20)(q13). The remaining four cell lines (UACC457, UACC647, 289, and 355) showed no karyotypic evidence of chromosome 20 abnormalities.

Karyotypes of the nine primary melanoma specimens used in this study are shown in Table 3. Like the cell lines, the majority (6/9) of the primary tumors had a modal ploidy of ~3N. Five of the nine primary tumor karyotypes (T94-274, T94-288, T95-002, T95-028, and T95-045) showed numeric abnormalities involving gain of the normal chromosome. Three primary specimens (T94-279, T94-280, and T94-046) showed no karyotypic abnormalities involving chromosome 20, and one specimen (T94-287) was karyotyped as -20.

FISH analysis

Results from FISH with the whole chromosome 20 paint probe are summarized in Tables 2 and 4. Chromosomes from peripheral blood lymphocytes donated by a normal male were used as a control in each FISH procedure and consistently showed the expected two hybridization signals for all the probes used in this study (Figure 8). Overall, 13/13 cell lines (100%) and 8/9 primary tumors (89%) showed extra copies of chromosome 20 DNA relative to modal ploidy.

Four melanoma cell lines (UACC827, UACC1022, UACC1065, and UACC457) showed low level copy number increases of chromosome 20, with a majority of the metaphases in each case possessing one extra signal relative to modal ploidy (Table 2). This was expected for UACC827 and UACC1022, as their respective karyotypes were both +20. The UACC457 and UACC1065 cell lines both had extra copies of chromosome 20 translocated to marker chromosomes.

In contrast, several cell lines showed high level copy number increases, ranging from two to four signals above what was expected for ploidy (Table 2). The UACC612

Table 1. Karyotype findings in 13 melanoma cell lines.

Case ID	Range	Modal Karyotype
UACC1529	46-65	60-63, X, +X, -Y, del(1)(p22), der(3)t(3;12)(q27;q11), +4, ?del(6)(q11), del(7)(q11-q21), add(7)(p13)t(2;7)(q12;q11), der(7)t(7;7)(p13;q11.2), -9, del(9)(p21), +11, add(12)(q22), -13, +15, +16, -18, +19, +20, -22, add(22)(q13), +7mar [cp17]
UACC1227	44-93	49-53, XY, +X, i(1)(q10), +7, add(8)(p23), -10,+15, +16, -20, -21, +3mar [cp17]
UACC827	43-65	55-62<3N>, XX, i(1)(q10), -5, add(5)(p13), del(6)(q13), +7, -11, add(11)(q21),-14, add(14)(q32), -18 x2, +20, +21, add(22)(p13), +5mar [cp13]
UACC457	57-69	66-69, XXY, i(1)(q10), +del(1)(q42),-4,del(6)(q13), -8, del(11)(q23), -13, -14, -15, +17, +3mar [cp13]
UACC1065	37-93	46, XX, del(5)(q21), +del(6)(q21), +7, ?del(7)(q11.2), -8, add(8)(p21), -9, der(9)t(1;9)(q21;p23), -10, del(10)(p13), -11, del(11)(q24), der(11)t(6;11)(p21;p13), -17, +20, -21, -22, [cp20]
UACC1022	42-115	59-62, XX, -Y, add(1)(q11), +add(1)(q11), del(1)(p22), add(3)(p22), +add(3)(p22), -5, +6, der(6)t(1;6)(p11;q27), +7, i(7)(p10), i(8)(q10), add(9)(p24), i(10)(q10), der(16)t(7;?;16)(p21;?;p13.3), -19, +20, +21, add(21)(p13), -22, -22, +7mar [cp13]
UACC1460	49-75	65-69, XXX, t(1;13)(p10;q10), +2, -4, add(6)(q11), +7, +7, +7, del(9)(p13)x2, +der(9)t(9;15)(p23;q11), -10, -12, -12, -13, -13, -14, -15, -16, -18, der(19)t(12;19)(q11;p11), -19, +20, +i(21)(q10)x2, +i(22)(q10), +22, +4mar [cp16]
UACC502	61-83	70-79, XXY, +1, +2, -4, del(6)(q16), add(6)(q23), -8, der(9)t(9;13)(q13;q11)x2, -12, -13, -14, der(16)t(4;16)(q11;p13)x2, +add(17)(q25), -18, -20, +9mar [cp31]
UACC612	62-83	70-71, XX, -Y, i(6)(p10), +8, -9, -12, -13, -16, -17, -18, +19, +20, +20, +5mar [cp10]
UACC647	63-112	72-77, XXY, +del(2)(p16), add(2)(q35), -4, -5, add(6)(q26), del(10)(q23), del(11)(q23), -13, -13, -14, +15, -16, -17, add(19)(q13), -21, +6mar [cp28]

Table 1 (continued). Karyotype findings in 13 melanoma cell lines.

Case ID	Range	Modal Karyotype
UACC375	65-143	123-136<6N>, XXXXY, -Y, -1, +2, -3, add(3)(q11)x2, -4, -4, der(4)t(4;22)(p11;q11)x2, -5, del(6)(q23?), +7, -8, -8, add(8)(q24), -9, der(9)t(9;21)(p11;q11), -10, -11, -11, -11, -12, -12, -12, der(12)t(12;22)(p11;q11), -13, -13, -13, -13, add(13)(p11), -14, -14, t(14;15)(q10;q10)x4, -15, -15, -15, -15, der(15)t(11;15)(q13;q22)x4, -16, -17, -17, -17, der(17)t(17;21)(p11;q11)x2, -19, add(19)(q13.3), -20, -20, add(20)(q13)x3, -21, -21, -21, -22, add(22)(q13), +7mar [cp11]
289	81-122	102-108<5N>, XXXX, -X, del(3)(p24.2)x2, -4, -5, del(6)(q23), +8, -9, -9, -9, del(10)(q11.2), der(11)t(3;11)(p10;q10), -13, -13, -14, -14, i(14)(q10), -17, -18, -18, -19, -21, -21, +5mar [cp11]
355	81-162	110-115, XXX, -X, -X, -1, add(2)(q37), -3, del(3)(p24), -4, -5, -5, del(5)(p22)x2, i(5)(p10), del(5)(q10), +6, del(6)(q23), del(7)(p10), +8, -9, -10, del(10)(q24), -13, -13, i(13)(q10), -14, -14, i(14)(q10)x2, +17, +18, -19, -22, -22, +3mar [cp10]

Table 2. Comparison of G-band karyotype and FISH (with a 20 whole chromosome paint probe) findings in 13 melanoma cell lines. Range of chromosome counts and modal numbers are presented as ploidy levels for reference to expected (based upon G-banding) and observed FISH signals of the modes (predominant populations).

Case ID	Ploidy Range	Modal Ploidy	Expected Chromosome 20 FISH signals	Observed FISH Signals
UACC1529	2N- to 3N-	3N-	4	4
UACC1227	2N- to 4N+	2N+	1	4
UACC827	2N- to 3N-	<3N->	4	4
UACC457*	2N+ to 3N	3N-	3	4
UACC1065*	2N- to 4N	2N	3	4
UACC1022	2N to 8N	3N-	4	4
UACC1460	2N+ to 3N+	3N-	4	4
UACC502*	3N- to 4N-	3N+	2	5 and 6†
UACC612	3N- to 4N-	3N+	5	6
UACC647*	3N- to 5N-	3N+	3	6 and 7†
UACC375	3N- to 6N+	<6N>	7	8
289*	4N- to 5N+	<5N->	5	7
355*	4N- to 6N+	5N-	5	6 and 7†

* Cell lines used in CGH screen.

† Equal percentage of metaphases counted for each signal number

Table 3. Karyotype findings in nine primary melanoma tumor samples.

Case ID	Range	Modal Karyotype
T94-274	42-123	67-70, XXY, +add(1)(q11), dup(3)(q22), dup(3)(q26), der(3)t(3;3)(q21;p21), -4, +add(6)(q11), +add(7)(q22), +8, -9, ins(9)(q12), -10, -11, -12, +13, der(15)t(15;22)(p11;q11)x2, -17, -18, +19, +20, +21, -22, -22, -22, +4mar [cp10]
T94-279	43-126	61-66, XX, -X, add(1)(q11), +add(1)(q11), -2, der(2)t(1;2)(q11;q37), +add(3)(p11), del(3)(q21), -4, +5, i(5)(p10)x2, der(6)t(6;7)(p10;q10), add(9)(p24), -10, -11, -12, i(13)q10)x2, -14, der(14)t(14;15)(p13;q11)x2, -16, -16, -17, -22, +5mar [cp16]
T94-280	39-55	51-55, XY, del(1)(q11), +del(1)(q11), der(3)t(1;3)(q11;p26), +5, i(6)(p10), +del(7)(p11), der(9)t(9;13)(p11;q11), +13, -16, +19, +add(19)(q13.4), +21, +4mar [cp14]
T94-287	55-110	71-73, XXY, +del(1)(p31), +add(4)(p11), +i(5)(p10), add(6)(q10), +add(6)(q10) +7, +7, add(9)(p22), +del(9)(p11)x2, del(12)(p12.1), -14, -16, -20, -21, add(21)(p13), -22, +4mar [cp7]
T94-288	39-80	67-70, XX, -Y, add(1)(q11), +del(1)(p12)x2, +del(1)(q12)x2, +der(?)t(?;1)(?;q11)x2, +add(1)(q11)x2, -2, +3, -4, -5, +7, +8, -9, -10, -11, +13, +13, -14, +15, -16, -17, +18, +der(19)t(6;19)(p21.2;p13.3), +20, +20, -21, -22, +2mar [cp8]
T95-002	44-106	92-100, XXX, -X, -2, add(3)(q27), -4, der(6)t(1;6)(q11;q11), add (6)(q11)x2, +der(6)t(1;6)(q11;q11), +7, +7, +7, +7, -10, -11, der(11)t(9;11)(q22;q14)x2. i(13)(q10), -14, -15, -16, +17, -18, +add(19)(p13.3)x2, +20, +13mar [cp15]
T95-028	55-86	61-65, XXX, +X, der(?)t(1;?)(q21;?), +add(1)(q11), -3, add(3)(q26.2)x2, -4, -5, -5, -6, add(6)(q12), add(7)(p11), +del(7)(q22)x2, -8, -9, -10, -11, add(12)(q22), -13, add(13)(p13), -17, -18, -18, -18, -19, -19, +20, -21, add(21)(p13), +22, +22, +7mar [cp18]
T95-045	64-112	96-106, XXY, -y, +3, add(5)(q35), add(5)(q11), add(6)(q11), +7, +7, +7, del(7)(q32), +8, -9, -10, -10, -13, add(13)(p11), del(17)(p13), -18, -18, +20, +20, +20, +20, +21,+9mar [cp13]
T95-046	53-133	72-78, XX, -X, +3, +4, -5, add(6)(q11), +7, +7, -8, del(9)(p22)x2, add(9)(p11), -10, -13, +15, +15, -16, -18, -18, +9mar [cp14]

Table 4. Comparison of G-band karyotype and FISH (with a 20 whole chromosome paint probe) findings in nine primary melanoma tumor specimens. Range of chromosome counts and modal numbers are presented as ploidy levels for reference to expected (based upon G-banding) and observed FISH signals of the modes (predominant populations).

Case ID	Ploidy Range	Modal Ploidy	Expected Chromosome 20 FISH signals	Observed FISH Signals
T94-274	2N- to 5N+	3N-	4	4
T94-279	2N- to 5N+	3N-	3	4
T94-280	2N- to 2N+	2N+	2	2
T94-287	2N+ to 5N-	3N+	2	5
T94-288	2N- to 3N+	3N-	5	4
T95-002	2N to 5N-	4N	5	6
T95-028	2N+ to 4N-	3N-	4	4
T95-045	3N- to 5N-	4N	8	6
T95-046	2N+ to 6N-	3N	3	4 and 5†

† Equal percentage of metaphases counted for each signal number

Figure 3 Chromosome 20 abnormalities detected by FISH in 3 melanoma cell lines. *Left:* GTG-banded chromosomes. *Right:* The same chromosomes after FISH with a 20 whole chromosome paint probe. A) UACC647 shows (from left) three normal chromosomes and multiple extra copies of chromosome 20 DNA in the form of markers. B) UACC375 shows (from left) two normal chromosomes, add(20)(q13), and der(20)add(20)(p13)add(20)(q13). C) UACC1227 shows (from left) two normal chromosomes, der(20)t(20;20)(p13;q12), and add(20)(p13).

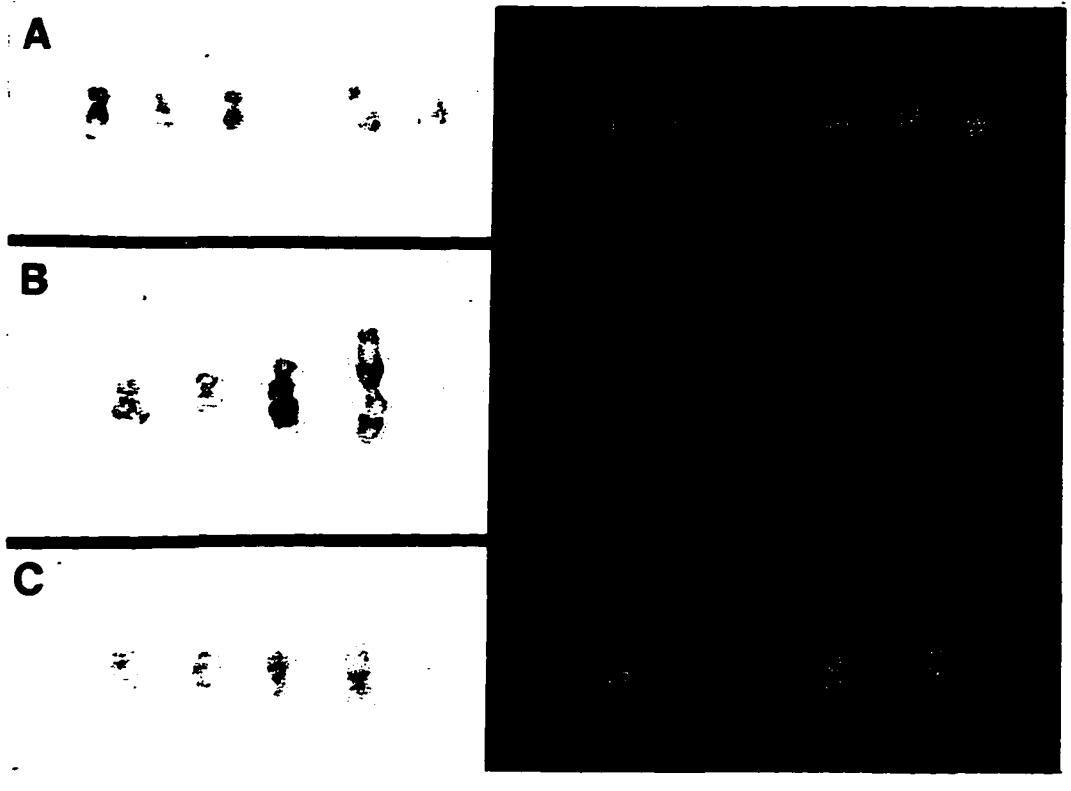


Figure 4 Representative metaphase from UACC502 showing (A) GTG-banded chromosomes and (B) FISH with a 20 whole chromosome paint probe. Karyotype analysis showed this cell line to be -20, yet FISH revealed chromosome 20 sequences translocated to der(2)t(2;20)(p10;q10), der(12)t(12;20)(q11;q11?), der(15)t(15;20) (q22;q11.1?), and marker chromosomes.

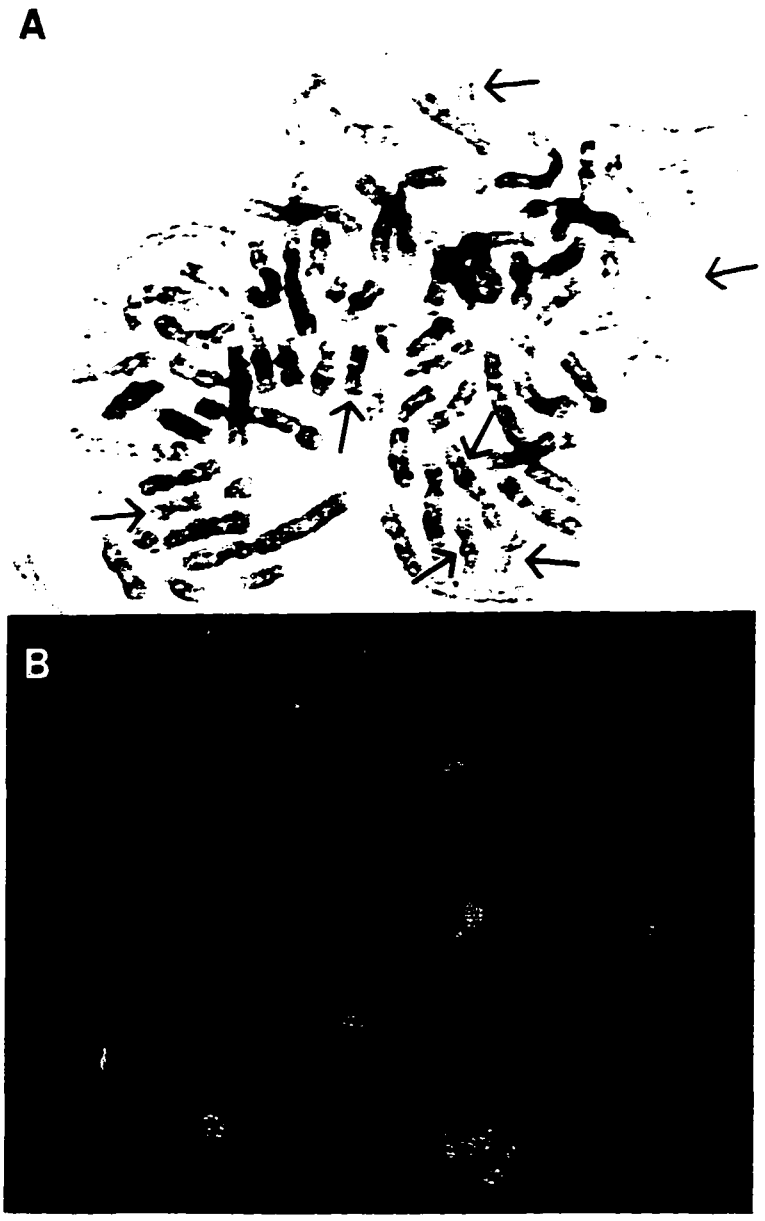


Figure 5 Chromosome 20 abnormalities detected by FISH in 2 primary melanoma specimens. *Left:* GTG-banded chromosomes. *Right:* The same chromosomes after FISH with a 20 whole chromosome paint probe. A) Case T94-287 shows normal chromosomes and a der(12)t(12;20)(p12.1;?) originally karyotyped as a del(12)(p12.1). B) Case T95-046 shows normal chromosomes, der(15)t(15;20)(p10;q10), and add(20)(q10).

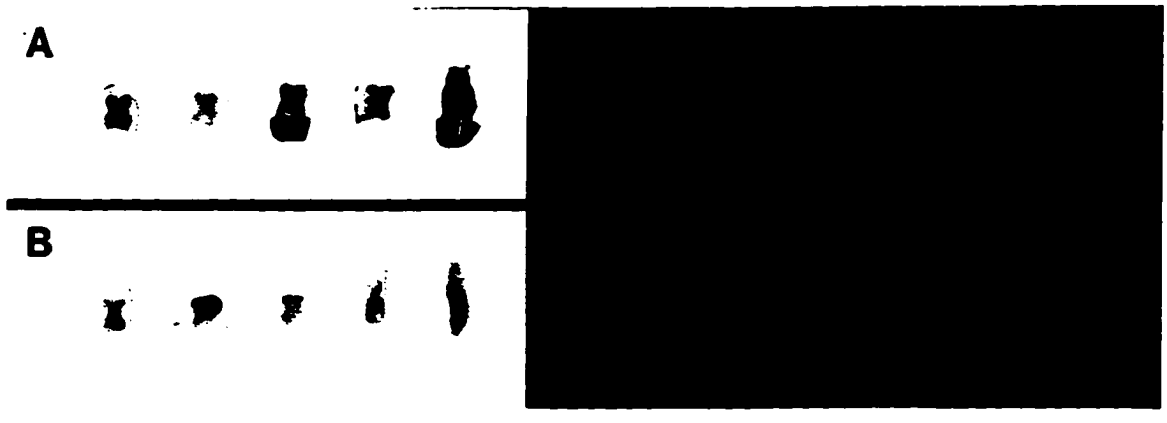


Figure 6 Results from FISH with the BT474 microFISH probe on 3 melanoma cell lines. Hybridization of this probe was used to confirm that translocated sequences identified by the 20 whole chromosome paint were derived from the 20q arm. A) UACC1227 cell line shows hybridization of probe to normal chromosomes (thin arrows) as well as der(20)t(20;20)(p13;q12) and add(20)(p13) chromosomes (thick arrows). B) UACC502 cell line shows hybridization of probe to normal chromosomes (thin arrows) as well as der(2)t(2;20)(p10;q10), der(12)t(12;20)(q11;q11?), der(15)t(15;20)(q22;q11.1?), and marker chromosomes (thick arrows). C) UACC647 cell line shows hybridization of probe to normal chromosomes (thin arrows) and markers (thick arrows).

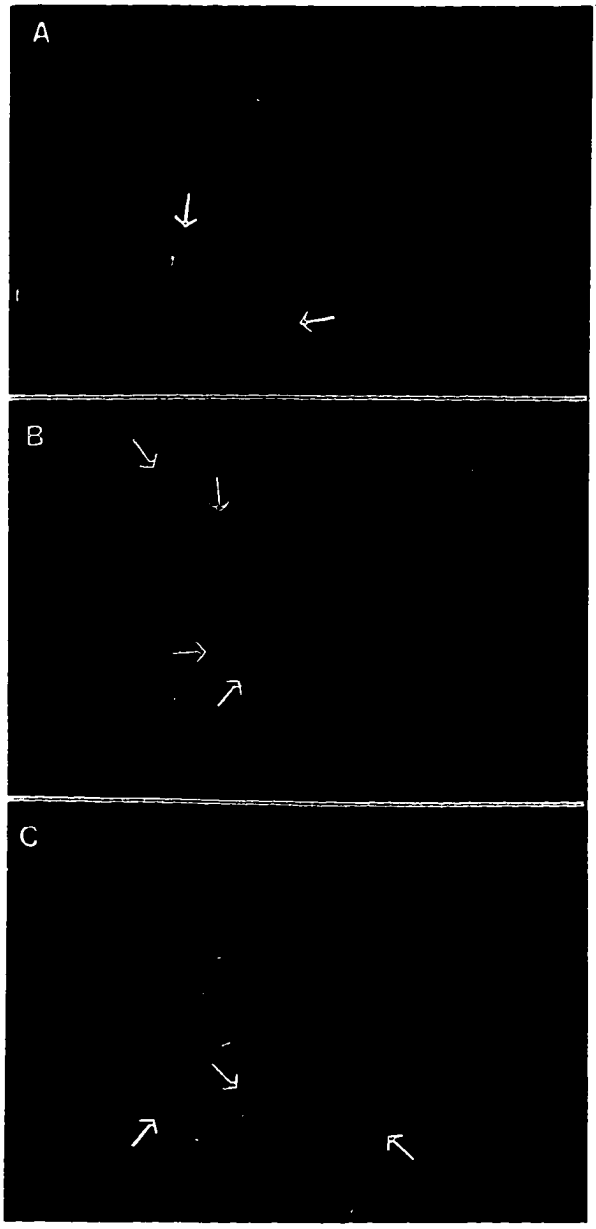


Figure 7 Results from FISH on UACC1227 with 20q band-specific P1 probes. A) Hybridization of the 20q11 band-specific probe (3916 P1). B) Hybridization of the 20q13 band-specific probe (112G8 P1). For both probes, signals corresponded to two normal chromosomes (thin arrows) and both derivative chromosomes (Figure 7A large arrows, Figure 7B inset).

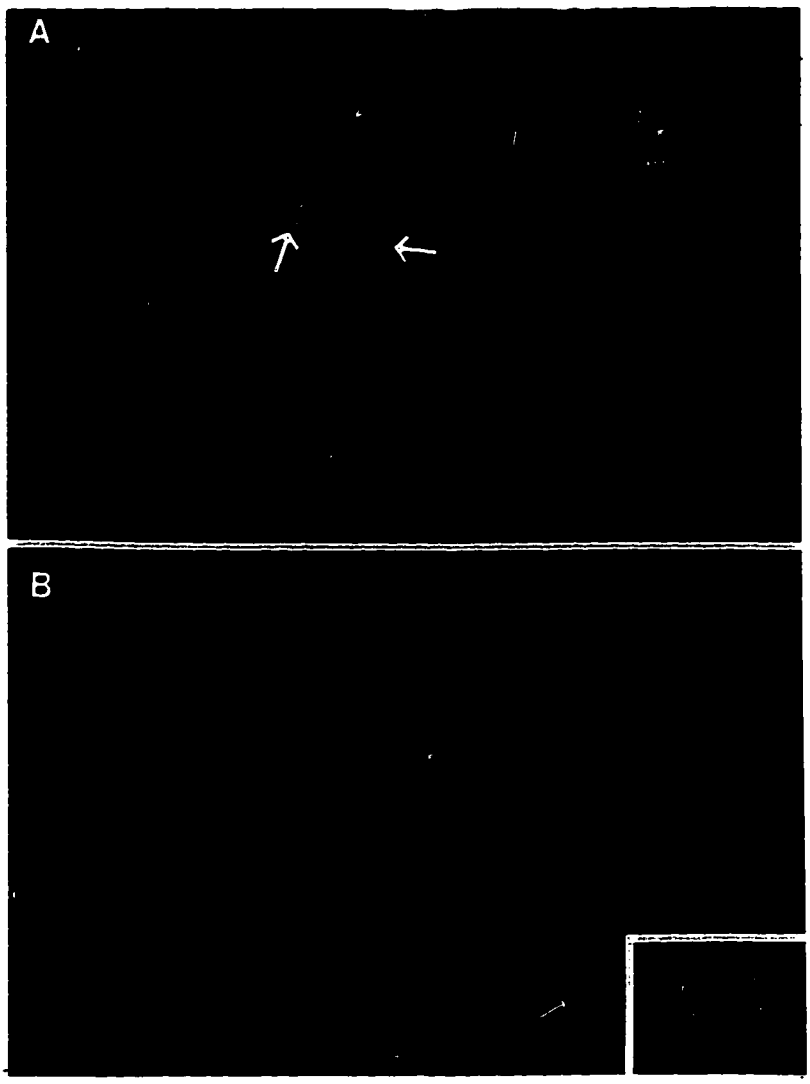
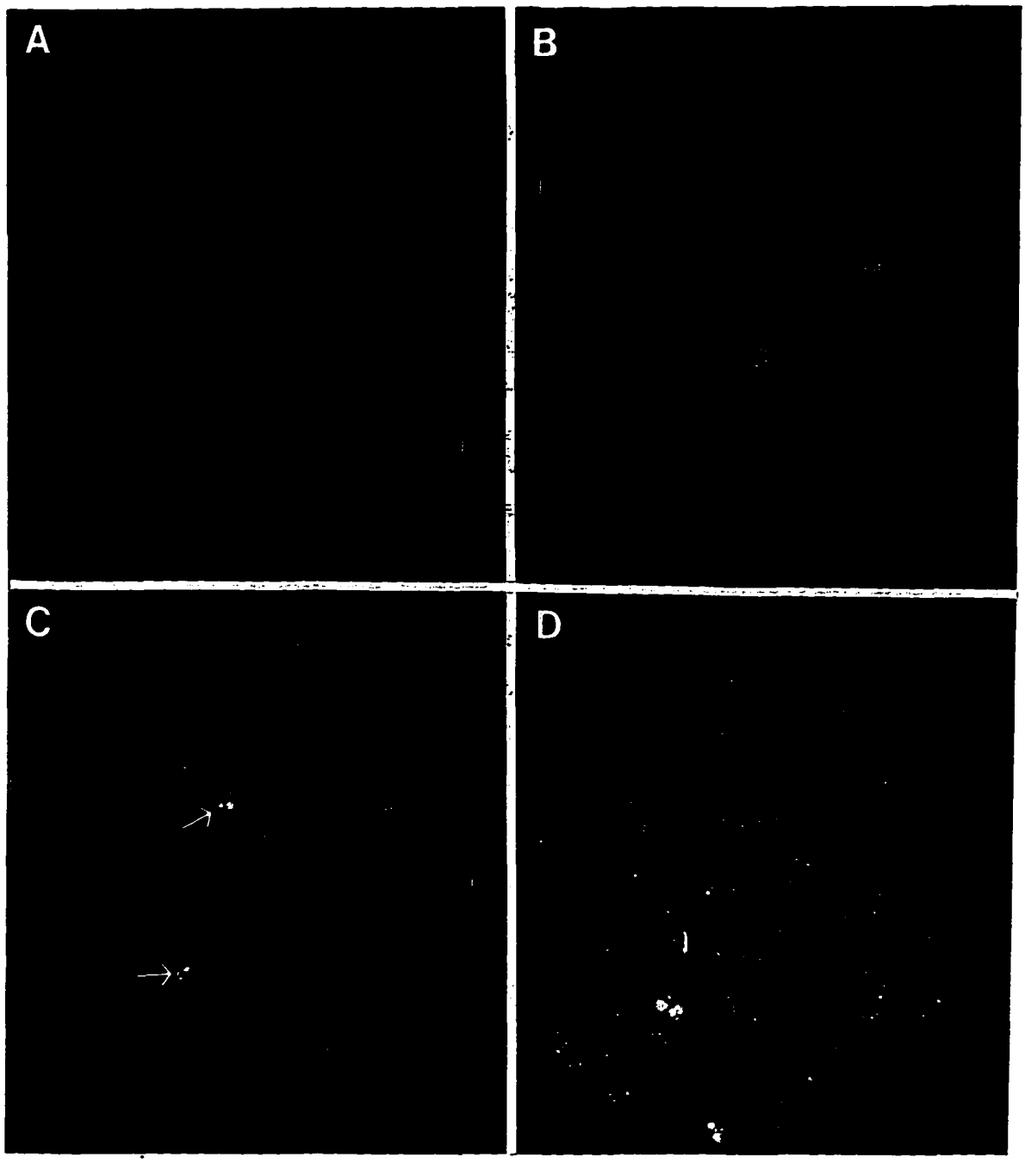


Figure 8 Hybridization of probes to normal blood lymphocytes (controls). All probes showed two signals as expected . A) Chromosome 20 whole chromosome paint probe B) BT474 microdissected probe specific for 20q11-13. C) 3916 P1 probe specific for 20q11. D) 112G8 P1 probe specific for 20q13.



cell line had two signals corresponding to the two extra copies of normal chromosome 20 described in the G-banded karyotype for this case: the third signal was due to hybridization of the probe to a marker chromosome. Similarly, the two extra signals observed in the 289 cell line corresponded to marker chromosomes, and 19/25 (76%) of the 355 cell line metaphases had one to two extra signals resulting from markers as well. Four cell lines with high copy number increases showed significant amounts of translocated chromosome 20 DNA. The UACC502 cell line had translocations to der(2)t(2;20)(p10;q10), der(15)t(15;20) (q22;q11.1?), and der(12)t(12;20)(q11;q11?), as well as to unknown markers (Figure 4). FISH on UACC502 with the BT474 microFISH probe confirmed that the translocated material was derived from 20q (Figure 6). The UACC375 cell line contained translocations to der(20)add(20)(p13)add(20)(q13), add(20)(q13), and unknown markers (Figure 3). Interestingly, the number of normal copies of chromosome 20 in UACC375 was 2-4/cell, and was thus outnumbered by the derivative copies of chromosome 20. The UACC647 cell line possessed three to four copies of chromosome 20 DNA translocated to marker chromosomes (Figure 3). Hybridization of the BT474 microFISH probe to these markers verified that they were composed of sequences from 20q (Figure 6). Of particular interest was the UACC1227 cell line which, in addition to containing signal-positive markers (1-2/metaphase), was shown by FISH to contain previously undetected der(20)t(20;20)(p13;q12) and add(20)(p13) chromosomes (Figure 3). Partial identification of the sequences comprising the add(20)(p13) was achieved with the BT474 microFISH probe, which revealed that a large portion of the additional material was derived from 20q11-q13 (Figure 6).

The nine primary tumor specimens analyzed segregated into three subgroups (Table 4). Three cases (T94-274, T94-280, and T95-028) showed agreement between the expected number of chromosome 20 signals and the observed number, with two of these specimens (T94-274 and T95-028) possessing extra copies of chromosome 20 relative to modal ploidy. In all three of these specimens, the observed signals corresponded solely to normal copies of chromosome 20. Four cases (T94-279, T94-287, T95-002, and T95-046) showed the observed number of signals exceeding the expected number. Cases T94-279 and T95-002 had extra signals corresponding to marker chromosomes. Case T94-287 was originally karyotyped as -20, but FISH revealed this to be incorrect: the specimen was in fact +20 and also showed a signal corresponding to a der(12)t(12;20)(p12.1;?) that had originally been karyotyped as del(12)(p12.1) (Figure 5). Case T95-046 showed extra signals corresponding to der(15)t(15;20)(p10;q10) and add(20)(q10) not previously

detected by standard cytogenetics (Figure 5): either one or both derivative chromosomes were present in any given metaphase, resulting in two equal sets of signal populations. In two cases, T94-288 and T95-045, the observed number of signals was less than the expected number: in both cases the karyotypes were reinterpreted according to the FISH data and changed to (+20) and (+20, +20) respectively.

Mapping by FISH with 20q band-specific P1 clones

As a first step toward defining the minimal region of amplification in melanoma, FISH analysis of the UACC1227 cell line was performed using bacteriophage P1 clones. These P1 clones, designated 3916 and 112G8, were derived from the BT474 breast cancer cell line and contain amplified, uncharacterized DNA sequences which map to bands 20q11 and 20q13, respectively (Figure 9). The UACC1227 cell line was chosen for more detailed analysis because it contains a 20q-derived add(20)(p13) as confirmed by FISH with the BT474 probe. G-band data for this chromosome suggested that the additional sequences may be due to an HSR (Figure 3). FISH analysis detected a total of six signals for each probe: two signals corresponded to hybridization of the probes to normal chromosomes, and the remaining four signals corresponded to hybridization of the probes to two locations each on the der(20)t(20;20)(p13;q12) and add(20)(p13) chromosomes (Figure 7). However, neither probe exhibited the extended hybridization signal consistent with the presence of an HSR, although both probes did reveal amplification of the 20q11 and 20q13 regions five-fold above the single signal predicted by the karyotype data for this cell line. Therefore, these results and those from the G-banding of this cell line (Figure 3) were interpreted as being indicative of an ABR on this arm of the chromosome. Because no ISCN nomenclature currently exists to describe ABRs, this chromosome was karyotyped as an add(20)(p13).

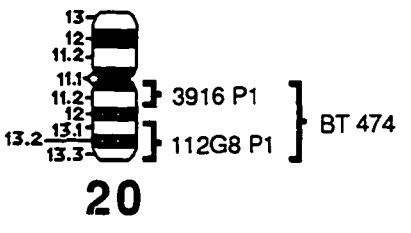


Figure 9 Diagram showing the chromosome 20 map locations of the P1 clones used for FISH analysis in this study.

DISCUSSION

Gene amplification is a manifestation of genomic instability in cancer. Many neoplasms exhibit increased DNA sequence amplification, including breast cancers, ovarian tumors, gliomas, and sarcomas (29, 30, 56, 57). However, little is known about the role of gene amplification in melanoma pathogenesis. Therefore, it is of considerable interest to identify chromosome regions harboring genes that may be targets for amplification in melanoma. This study illustrates the potential of molecular cytogenetic techniques for rapidly identifying genomic sites that may harbor putative oncogenes.

The results of FISH with the whole chromosome 20 paint probe on the cell lines screened by CGH confirm our observation that 20q is amplified in melanoma. The CGH profiles for all seven of the cell lines that were screened showed amplification of 20q, even in cell lines where the karyotype data indicated no chromosome 20 abnormalities (UACC457, UACC647, 289, and 355) or loss of 20 (UACC502). Our FISH data shows that in six out of seven of the CGH-screened cell lines, extra signals were observed in relation to both modal ploidy and expected chromosome 20 FISH signals. Five of the six cell lines (UACC457, UACC1065, UACC647, 289, and 355) contained the extra signals in marker chromosomes, and the sixth (UACC502) possessed multiple signals corresponding to derivative chromosomes resulting from translocation of chromosome 20q to chromosomes 2p, 12q, and 15q. UACC502 also possessed various markers that were signal-positive for the probe. The CGH profiles of UACC502 and UACC647 exhibited very high levels of 20q DNA copy number increases, and our FISH results showed that these two cell lines possessed the highest number of extra signals relative to ploidy when compared with the other cell lines included in this study.. One cell line screened by CGH, UACC1509, was not used in this study due to extremely low mitotic index and subsequent lack of karyotype data. Overall, the agreement between the CGH and FISH profiles for this subset of six cell lines suggests that a gene(s) important to melanoma pathogenesis is located on 20q.

Since FISH on the abovementioned cell lines was conducted to confirm our CGH observations, it was of interest to analyze other cell lines not previously screened by CGH in order to assess the overall extent of 20q amplification in our cell lines. FISH performed on seven cell lines not screened by CGH yielded similar observations in that all of these lines contained extra signals in relation to modal ploidy. However, unlike the CGH-screened cell lines, four of these lines (UACC1529, UACC827, UACC1022, and

UACC1460) carried the expected number of chromosome 20 FISH signals as described by their respective G-banded karyotypes. Interestingly, all four of these cell lines had a modal ploidy of ~3N and carried one extra normal copy of chromosome 20, perhaps indicating that gain of the whole chromosome relative to ploidy is an early event in the transformation of melanoma cells to a malignant phenotype. The UACC375 cell line was unique among the cell lines included in this study because the majority of its copies of chromosome 20 were present as derivative chromosomes resulting from translocations with unknown partners: normal copies of chromosome 20 ranged between 2-4/metaphase, while three to five copies of the add(20)(q13) and two to four copies of the der(20)add(20)(p13)add(20)(q13) were typically present. A breakpoint at 20q13 was also observed in one of the two derivative chromosome 20s present in the UACC1227 cell line. These findings are consistent with a recent study of 158 metastatic melanoma specimens conducted by Thompson et al. which found that the only recurring breakpoint on chromosome 20 was 20q13 (26, see also Figure 2B). However, the very low incidence of breakpoints involving 20q13 in malignant melanoma suggests that translocations resulting in fusion proteins (such as the bcr-abl protein formed by the Ph^1 chromosome) derived from genes in this region are not significant as mechanisms for conferring a malignant phenotype to melanocytes. In general, this subset of cell lines showed a lesser overall degree of chromosome 20 amplification than the cell lines screened by CGH because of a lower occurrence of marker chromosomes, but the agreement between the results obtained from each subset suggests that chromosome 20 amplification is a common event in metastatic melanocytic lesions.

To ensure that the chromosome 20 abnormalities we observed were not due to the effects of *in vitro* tissue culture selection pressure, metaphase spreads from surgically resected melanomas were analyzed by FISH for extra copies of chromosome 20 DNA. The primary tumor specimens used for this study were not the same specimens that we evaluated via CGH due to insufficient sample quantity. However, amplification of chromosome 20 DNA was observed in eight of the nine primary specimens chosen for this analysis. Amplified chromosome 20 DNA was observed as additional copies of the normal chromosome in 46% of the primary tumors, marker chromosomes in 22%, and translocations in 22%. Unfortunately, the paucity of primary tumor material available for FISH analysis made it impossible to further characterize the origin of extra chromosome 20 DNA in three of the specimens displaying markers or translocations: in the fourth (T95-046) the extra material was identified as 20q via G-banding. However, our CGH data

from the other archival specimens (54) coupled with our findings in this study strongly suggest that these three primary specimens may have their additional chromosome 20 sequences derived from the 20q arm. In general, the similarities between the frequency and morphology of chromosome 20 abnormalities detected in our cell lines and primary tumors suggest strongly that the amplification of chromosome 20q observed in the cell lines is not merely an artifact of *in vitro* tissue culture selection. Our cell lines can thus serve as an accurate model system in which future studies can be conducted to elucidate the most consistent region of chromosome 20 amplification in melanoma.

The co-occurrence of chromosome 20 anomalies with the other most commonly observed numeric abnormalities in melanoma, namely +7, -9, and -10, has not been described in any other studies to date. For example, it has been shown in melanoma that loss of one chromosome 10 is often accompanied by gain of one or more copies of chromosome 7, and that this coincidence most frequently occurs during the later stages of melanoma pathogenesis (58). The combined results of this study did not reveal any preferential association of chromosome 20 with chromosomes 7, 9, or 10: gain of 7 coincided with gain of 20 in 6/22 specimens as did loss of 10 and gain of 20, while loss of 9 and gain of 20 coincided in 8/22 specimens. Since only malignant melanomas were studied in this series, the order of occurrence of chromosome 20 numeric changes relative to those of chromosomes 7, 9, and 10 in the different stages of tumorigenesis cannot be established. The analysis of a subset of 49 near-diploid metastatic melanomas by Thompson et al. shows that chromosome 20 was gained or lost with nearly equal frequency in these tumors, which suggests that chromosome 20 abnormalities may not play a significant role in early metastatic transformation (26, see also Figure 2A). Therefore, further studies using earlier stage melanocytic lesions (i.e. atypical nevi, dysplastic nevi, and Stage 1 melanoma) will be needed to help clarify the timing of chromosome 20 abnormalities during melanoma progression.

Cutaneous melanoma tumors account for close to 90% of all melanomas, with approximately 10% of these arising in individuals with a familial background and the other 90% being sporadic in origin (59). However, the genetic similarities between familial and sporadic melanomas remain poorly described to date. The most thoroughly described locus common to both types of melanoma is chromosome 9p21: a number of recent studies focusing on both familial and sporadic melanocytic lesions (7, 60-61) have demonstrated the occurrence of deletions and other mutations in the p16 gene located on 9p21. Although all of the specimens used for this study were sporadic in origin, our findings raise the

possibility that there is a gene or genes on the long arm of chromosome 20 that may also be involved in familial melanomas. Therefore, future experiments should include examining the genomes of familial melanoma kindreds for chromosome 20 anomalies. This in turn would provide a more complete picture of the overall role of chromosome 20 anomalies in melanoma development.

Gene amplification in solid tumors can be manifested as ABRs, HSRs, or extra-chromosomal material in the form of DMs. As a first step toward identifying potential candidate genes on 20q involved in melanoma pathogenesis, mapping studies were initiated with specific P1 clones. This series of studies focused on the UACC1227 cell line because it showed direct cytological evidence for gene amplification at 20q in the form of an ABR that manifested as an add(20)(p13). FISH analysis with 20q11 and 20q13 band-specific P1 probes showed that although the DNA sequences for 20q11 and 20q13 were retained within the amplicon, the exact composition of the ABR could not be determined using these probes alone. Additional FISH analysis with a 20q12 band-specific probe and 20p arm specific probes is required to resolve this question. Interestingly, Guan et al. have recently isolated amplified cDNAs from a breast cancer cDNA library using the BT474 microdissection probe and designated them (amplified in breast cancer) AIB1, AIB3, and AIB4: these cDNAs are of interest because they map to 20q11 (AIB3, AIB4) and 20q12 (AIB1)(62). The P1 clones used in the present study contain the AIB3 and AIB4 genes (3916 P1) as well as DNA sequences from the 20q13 region found to be most consistently amplified in breast cancer (112G8 P1)(29). Our results suggest strongly that AIB3, AIB4, and/or gene(s) in 20q13 are also amplified in malignant melanomas, and therefore might be attractive candidate genes important to melanoma pathogenesis . However, further molecular cytogenetic studies on this and other cell lines with the P1 clones included in this study and additional P1 clones specific for 20q12 are required to fully resolve this question. Additionally, molecular genetic studies (i.e. Northern and Southern blot analyses, gene transfer studies, etc.) are needed to confirm this finding.

Other genes existing on 20q besides those described above are also potential candidates for the putative oncogene in melanoma pathogenesis. One potential gene target for amplification in melanoma may be E2F1, a transcriptional activator which has been mapped to the 20q11 region (31) and is important for the transcription of cell cycle-regulated genes such as c-*myc*, N-*myc*, cdc2, cyclin A, the retinoblastoma susceptibility protein (pRb), dihyrofolate reductase (DHFR), thymidine kinase, thymidylate synthetase, and DNA polymerase α (rev. in 31). A central role for E2F in cell growth control is

suggested by the fact that it forms complexes with underphosphorylated forms of pRb and the related p107 protein, which both inhibit transcriptional *trans*-activation of gene expression normally mediated by E2F (31). In erythroleukemias, increased copies of E2F1 have been demonstrated using FISH and other molecular genetic techniques (31). Additionally, preliminary molecular genetic studies conducted in our laboratory show increased copies of E2F1 in melanoma cell lines and surgically resected melanoma tumors (data not shown). It is possible that overexpression of E2F1 could confer a tumorigenic phenotype by overwhelming the pRb and p107 inhibitory mechanisms. pRb is located at 13q14.1 (18), and it is interesting to note that in this study, loss of 13 relative to gain of 20 occurred in 19/22 specimens: not only is this co-occurrence significantly higher than that for chromosomes 7, 9, and 10, it was detected karyotypically in 15 of these 19 cases and by FISH in the remaining four. This strongly suggests that these genes or others on 13 and 20 are interacting to produce a malignant phenotype in melanocytic neoplasms. In future studies, it may be of interest to investigate the relationship between chromosomes 13 and 20 in melanoma tumor genomes to discern whether whole chromosome copy number changes are the mechanism causing tumorigenesis or if other processes such as microdeletions, point mutations, promoter or enhancer sequence alterations, or alternative splicing of candidate genes on these chromosomes are responsible.

In summary, gene amplification is an important mechanism for up-regulating genes that contribute to tumorigenesis. In the present study, we have used state-of-the-art molecular cytogenetic techniques - chromosome microdissection, CGH, and FISH - to implicate the long arm of chromosome 20 in melanoma pathogenesis. Furthermore, initial mapping studies with the UACC1227 melanoma cell line suggest that the minimal region of consistent amplification in melanoma may be located in 20q11 or 20q13. Finally, our study suggests that AIB3, AIB4, E2F1, or genes in 20q13 represent attractive candidate genes that might contribute to the development of malignant melanoma.

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