Neoplasia • Vol. 7, No. 4, April 2005, pp. 303–311 303

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Seven Novel and Stable Translocations Associated with Oncogenic Gene Expression in Malignant Melanoma[1]

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Abstract

Cytogenetics has not only precipitated the discovery of several oncogenes, but has also led to the molecular classification of numerous malignancies. The correct identification of aberrations in many tumors has, however, been hindered by extensive tumor complexity and the limitations of molecular cytogenetic techniques. In this study, we have investigated five malignant melanoma (MM) cell lines from at least three different passages using high-resolution R-banding and the recently developed methods of comparative genomic hybridization and multicolor or multiplex fluorescence *in situ* hybridization. We subsequently detected nine consistent translocations, seven of which were novel: dic(1;11)(p10;q14), der(9)t(3;9)(p12;p11), der(4)t(9;4;7)(q33::p15-q23::q21), der(14)t(5;14)(q12;q32), der(9)t(9;22)(p21;q11), der(19)t(19;20)(p13.3;p11), der(10)t(2;12;7;10)(q31::p12→pter::q11.2→q31::q21), der(19)t(10;19)(q23;q13), and der(20)t(Y;20)(q11.23;q13.3). Furthermore, using the human HG-U133A GeneChip, positive expression levels of oncogenes or tumor-related genes located at the regions of chromosomal breakpoints were identified, including AKT1, BMI1, CDK6, CTNNB1, E2F1, GPNMB, GPRK7, KBRAS2, LDB2, LIMK1, MAPK1, MEL, MP1, MUC18, NRCAM, PBX3, RAB22A, RAB38, SNK, and STK4, indicating an association between chromosomal breakpoints and altered gene expression. Moreover, we also show that growth of all five cell lines can be significantly reduced by downregulating *CDK6* gene expression with small interfering RNA (siRNA). Because the majority of these breakpoints have been reported previously in MM, our results support the idea of common mechanisms in this disease.

Neoplasia (2005) **7,** 303–311

Keywords: Melanoma, oncogenic gene expression, translocation, CDK6, siRNA.

Introduction

Malignant melanoma (MM) is a fatal disease once metastasis has occurred and a dramatic increase in incidence has been recorded [1]. Despite successful identification of molecular mechanisms in many malignancies using cytogenetic data, MM still remains a challenging entity [2,3]. To date, a recurrent involvement of chromosomes 1, 3, 6, 7, 9, 10, and 11 in MM has frequently been reported, although common cytogenetic aberrations with molecular characterization have yet to be defined [3,4].

Only recently, molecular cytogenetics has been shown to be effective in revealing complex cytogenetic events, even in MM [5]. For example, using fluorescently labeled DNA probes, fluorescence *in situ* hybridization (FISH) allows DNA copy number and the chromosomal location of a specific gene locus to be analyzed, thus revealing detailed information regarding translocations or DNA copy number changes [6]. The information provided by FISH is, however, limited to the precise location of the probe, leaving the majority of genomic changes undetected. In spite of this, multicolor or multiplex fluorescence *in situ* hybridization (M-FISH) and comparative genomic hybridization (CGH) identify cytogenetic abnormalities throughout the entire genome [7]. Using M-FISH, each human chromosome can be distinguished by a distinct color combination, allowing complex rearrangements to be identified [8]. CGH can determine the DNA copy number by hybridization of labeled tumor DNA (test) and normal DNA (reference) to normal metaphase

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[1]This work was supported by the Jubiläumsfonds der Österreichische Nationalbank (grant no. 9961) and the "EU COST B19 action."

Received 26 July 2004; Revised 17 December 2004; Accepted 4 January 2005.

DOI 10.1593/neo.04514

chromosomes [9]. The ratios of fluorescence intensities on the chromosomes then reflect the copy number changes of corresponding sequences in the tumor DNA. Both methods do not require previous knowledge of genetic aberrations to evaluate the extent of genetic gains or losses in the genome. To gain the maximum benefit of whole genome analysis for structural as well as numeric aberrations in MM cells, we have combined M-FISH and CGH with CDD banding.

We then asked whether oncogenes located at these consistently found breakpoints were dysregulated in cells harboring the breakpoints. To gain the maximum benefit of whole genome analysis for chromosomal aberrations, as well as functional analysis on the mRNA level in MM cells, we have combined our comprehensive cytogenetic data with DNA microarray analysis. Strikingly, our results show that the majority of stable breakpoints identified are associated with increased oncogene expression levels when compared to MM cell lines without the aberrations, suggesting a direct relationship between chromosome aberrations and oncogenic development.

Materials and Methods

Cell Lines

The human melanoma cell lines A375, MelJUSO, and Skmel 28 were obtained from the ATCC (Manassas, VA), and 518A2 and 607B cells were a kind gift from Dr. P. Schrier (University of Leiden, Leiden, The Netherlands). All cells were cultured in DMEM (Gibco BRL, Paisley, UK) supplemented with 10% FCS and a 1% antibiotic–antimycotic mix (Gibco BRL) in a humidified 5% CO_2–95% ambient air atmosphere at 37°C.

R-banding

Cells were harvested and fixed as previously described [10]. To obtain the most accurate information and high resolution of banding patterns, we applied chromomycin/distamycin/DAPI (CDD) staining [11]. This method provides a high quality of reverse (*R*-) and 4′–6′-diamidino-2-phenylindole-2HCl (DAPI) banding in a single experiment. The karyotypes were classified using the International System for Human Cytogenetic Nomenclature ISCN 1995 as recommended by the International Standing Committee of Cytogenetic Nomenclature.

FISH and M-FISH

FISH was performed as described elsewhere [6]. As probes, BAC clone RP 11-888H2 containing the *CDK6* gene and RP11-893C3 (4q21.21) was used to prove the involvement of the *CDK6* gene in the marker t(4;7;9) in the 518A2 cell line.

For M-FISH, metaphase spreads were prepared overnight at room temperature and analyzed with M-FISH using the SpectraVysion 24-color karyotyping assay according to the manufacturer's protocol (Vysis, Downers Grove, IL). Metaphase images were captured with an epifluorescence microscope (Zeiss, Göttingen, Germany) equipped with a charged-coupled device (CCD) camera (Photometrics, Tucson, AZ) and using single-bandpass filters (SpectraVysion optical filters; Vysis) corresponding to the fluorophores SpectrumGold, SpectrumFRed, SpectrumAqua, SpectrumRed, SpectrumGreen, and the fluorescent DAPI counterstain. Further image processing and 24-color karyotyping were performed with the SpectraVysion Imaging System (Vysis). At least 10 metaphase spreads from each cell line were analyzed for M-FISH karyotyping.

CGH

Genomic DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and stored at −20°C. Normal male reference DNA was obtained from Promega (Mannheim, Germany). Amplification by DOP polymerase chain reaction (PCR) and labeling of DNA samples were performed essentially as previously described [12], and 1–2 μl of the DOP-PCR product was used for PCR labeling with Dig-11-dUTP (tumor DNA) or Bio-16-dUTP (reference DNA) (Roche, Mannheim, Germany). Labeling was carried out in 50-μl reaction mixtures comprising 200 μM each of dATP, dCTP, and dGTP each; 160 μM dTTP; 40 μM Dig-11-dUTP or Bio-16-dUTP; 1% W1 detergent; 2 μM degenerate primer; and 0.1 U/μl Super Taq DNA Polymerase in TAPS buffer (25 mM TAPS, 50 mM KCl, 2 mM $MgCl_2$, and 2.88 μM β-mercaptoethanol) for 5 minutes at 95°C, 30 cycles of 1.5 minutes at 94°C, 1.5 minutes at 58°C, and 3 minutes at 72°C, followed by a final extension at 72°C for 8 minutes. Equal amounts of tumor DNA and reference DNA-PCR products, along with a sufficient amount of human *Cot*I DNA (Roche), were hybridized in Hybridisol (QBIOgene, Carlsbad, CA) on normal human metaphase slides (Vysis). For probe detection, a 1:200 dilution of anti–Dig-FITC (Sigma, St. Louis, MO) antibody and a 1:100 dilution of anti–Biotin-Cy3 antibody (Sigma) were employed. After mounting with DAPI containing antifade solution (Vector Laboratories, Inc., Burlingame, CA), images were captured with a Leica DMRXA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate epifluorescence filters and a COHU CCD camera. CGH profiles were analyzed using Leica QFISH and the Leica QCGH software. Ten to 15 metaphases per case were analyzed to create the median ratio profile. Increases and decreases in DNA sequence copy numbers were defined by tumor-to-reference ratios of >1.2 and <0.8, and amplifications at ratios >1.4, respectively. These reference values were established before the study by CGH analysis of normal DNA samples from different sources as a specificity control, as well as different mixtures of male and female normal DNA and analyses of X-chromosome material as a sensitivity control. The mean green-to-red ratio of normal DNA was always between 0.8 and 1.2 on all chromosomes, with the exception of chromosomes 19, 22, and 1p32-pter [13], which were not included in the analyses.

Gene Expression Profiling

Total RNA was isolated from MM cell lines using the Qiagen RNeasy Mini Kit (Qiagen) and subjected to DNA