Molecular Characterization of a t(9;12)(p21;q13) Balanced Chromosome Translocation in Combination with Integrative Genomics Analysis Identifies C9orf14 as a Candidate Tumor-Suppressor

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A large number of nevi (LNN) is a high risk phenotypic trait for developing cutaneous malignant melanoma (CMM). In this study, the breakpoints of a t(9;12)(p21;q13) balanced chromosome translocation were finely mapped in a family with LNN and CMM. Molecular characterization of the 9p21 breakpoint identified a novel gene C9orf14 expressed in melanocytes disrupted by the translocation. Integrative analysis of functional genomics data was applied to determine the role of C9orf14 in CMM development. An analysis of genome-wide DNA copy number alterations in melanoma tumors revealed the loss of the C9orf14 locus, located proximal to CDKN2A, in approximately one-fourth of tumors. Analysis of gene expression data in cancer cell lines and melanoma tumors suggests a loss of C9orf14 expression in melanoma tumorigenesis. Taken together, our results indicate that C9orf14 is a candidate tumor-suppressor for nevus development and late stage melanoma at 9p21, a region frequently deleted in different types of human cancers. This article contains Supplementary Material available at http://www.interscience.wiley.com.

INTRODUCTION

Cutaneous malignant melanoma (CMM) is a potentially fatal type of skin cancer with increasing incidence and mortality world wide (Rigel, 1996; Jemal et al., 2003). A major etiological factor in the development of CMM is sunlight exposure (Pho et al., 2006). In addition, epidemiological studies have revealed that of a number of phenotypic traits, the highest risk of developing CMM is conferred by the presence of a large number of nevi (LNN) (Swerdlow and Green, 1987; Grob et al., 1990; Bataille et al., 1996; Briollais et al., 2000). As with CMM, sunlight exposure is also the major etiological factor in nevus ontogenesis. However, the association between sunlight exposure, CMM, and nevus development is relatively complex. In addition to environmental factors, there are known and unidentified genetic factors that contribute to both phenotypes either independently or in association. Thus, total nevi counts and nevus density show familial aggregation (Goldgar et al., 1991; Duffy et al., 1992). Familial aggrega-

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tion of LNN and CMM has also been described (Bahuau et al., 1997; Briollais et al., 2000) although mutations in known melanoma susceptibility genes do not always co-segregate with LNN (Puig et al., 1997; Hayward, 2000). In approximately 30% of cases, a histological association exists between CMM and a precursor nevus (Seykora and Elder, 1996; Bevona et al., 2003). Accordingly, \textit{CDKN2A} and \textit{BRAF} mutations have been associated with the development of both nevus and CMM (Pollock et al., 2003; Kumar et al., 2004; James et al., 2005). On the basis of these observations, the existence of diverse but partially overlapping genetic and molecular mechanisms for the development of nevus and CMM has been proposed (Rivers, 2004).

Cytogenetic analysis identified a t(9;12) (p21;q13) balanced chromosome translocation in a family with LNN and CMM. Fine mapping of the breakpoints identified a novel gene (\textit{C9orf14}) disrupted by the translocation at 9p21. Molecular characterization of the translocation breakpoint and integrative analysis of functional genomics data or “integrative genomics” indicate that \textit{C9orf14} is a candidate tumor-suppressor for nevus development and late stage melanoma.

**METHODS**

**Patients**

Study protocol was approved by the Institutional Review Board of the Hospital Clinic of Barcelona. All family members gave written informed consent to participate in the study.

**Southern and Pulse-Field Gel Electrophoresis Analyses**

Genomic DNA was extracted from whole blood using standard protocol. High molecular weight DNA was purified from agarose-embedded lymphocytes by proteinase K digestion and electrophoresed using a CHEF-DRII Mapper (Bio-Rad). Conventional alkaline transfer of DNA was used. PCR products were gel-purified using the QIAGen Gel Band Purification Kit and then labeled using the Megaprime DNA Labeling System (GE Healthcare).

**Cytogenetic and Fluorescence In Situ Hybridization Analyses**

Blood cells were cultured and harvested according to standard cytogenetic protocols. Karyotypes are described according to ISCN. For fluorescence in situ hybridization (FISH) analysis, genomic clones were purchased from CEPH (France), the Human Genome Mapping Project Resource Centre (United Kingdom), Research Genetics (USA), and the BACPAC Resource Center (USA). YAC's inter-Alu PCRs and BAC/PAC DNA mini preparations (QIAGen) were labeled with either biotin-16dUTP or digoxigenin-11dUTP (Roche Applied Science) by a standard nick-translation reaction. Slides were visualized under a fluorescence microscope (AH3, Olympus) with appropriate filters. Images were analyzed using the Cytovision system (Applied Imaging Ltd.).

**DNA Copy Number Data Analysis**

Data for DNA copy number alterations in primary melanoma tumors was downloaded from the Gene Expression Omnibus (GEO) record GSE2631 (Curtin et al., 2005). This data set contains log\textsubscript{2} ratios of DNA analysis of tumor versus normal tissue samples. The Stats and Graphics packages in R (www.R-project.org) were used for clustering analysis.

**Microarray Gene Expression Data Analysis**

The NCI60 cancer cell lines expression data set analyzed was GeneLogic Affymetrix U95D (Scherf et al., 2000), which contains a single \textit{C9orf14} probe (69061_at; AI078569). This data set contains normalized and scaled expression values. The Stats and Graphics packages in R (www.R-project.org) were used for clustering analysis. Raw expression data from normal skin, keratinocytes, melanocytes, nevi, and melanomas was published elsewhere (Smith et al., 2005) and deposited in GEO record GSE4587. Short-term cell cultures were used by the authors to minimize expression artifacts. We downloaded the raw data and analyzed it using the Robust Multi-array Average algorithm (Irizarry et al., 2003) and the LIMMA package (Smyth, 2004). Differential gene expression between classes was then assessed using the Student's \(t\) test. The ovarian expression data set was published elsewhere (Lu et al., 2004) and used the same methodology. Raw expression data of different normal human tissue samples was downloaded from the GEO record GSE3526 and analyzed using the same methodology.

**\textit{C9orf14} Cloning and Gene Structure Analysis**

EST clones were purchased from the Human Genome Mapping Project Resource Center (United Kingdom) and the Resource Center within the German Human Genome Project (RZPD). The SL.2NbHM normalized normal melanocyte
C9orf14 cDNA library was purchased from Research Genetics. Human Multiple Tissue Northern (MTN) and Marathon-Ready cDNAs were purchased from Clontech-Takara. Total RNA from normal skin biopsies and cell lines (normal human epidermal melanocytes (PromoCell), HeLa, HepG2, NP29, and SHY) were extracted with TriPure Isolation Reagent (Roche Applied Science). Total RNA was reverse transcribed using random hexamers and Superscript II (GIBCO-BRL, Invitrogen) according to the manufacturer’s instructions. RT-PCR reactions were carried out with Advantage-GC 2 Polymerase Mix (Clontech-Takara) and did not exceed 40 cycles. All RT-PCR reactions were performed at least twice for each cDNA sample using GAPDH as positive control (primers GGTGAAGGTCGGAGTCAACG and CAAAGTTGTCATGGATGACC). Purification of small RNAs was carried out using the mirVana miRNA Isolation Kit (Ambion). Fifty micrograms of total RNA were then used for purification and Northern blotting.

**C9orf14 Protein Localization Study**

Protein expression analysis was carried out using the Gateway system (Invitrogen). Briefly, the PCR-amplified open reading frame (ORF) sequence was cloned by site-directed recombination into the pENTR201 entry vector and then transferred into the pT-Rex-DEST31 expression vector. Telomerase-immortalized human mammary epithelial cells (HME/TERT) (DiRenzo et al., 2002) growing on glass cover slips were transfected with Opti-MEM Reduced Serum Medium and Lipofectamine 2000 (Invitrogen), and then fixed in cold methanol for 10 min followed by cold acetone for 1 min. Staining was performed overnight at 4°C using a 1:1,000 dilution of an anti γ-tubulin (TUBG1) (Sigma). PBS-Tween 0.02% containing 4% fetal bovine serum was used in antibody incubations and PBS-Tween 0.02% was used for washes. Alexa-596 conjugated anti-mouse secondary antibody (Invitrogen, Molecular Probes) was used for detection.

**RESULTS AND DISCUSSION**

Cytogenetic analysis identified a t(9;12) (p21;q13) balanced translocation in three members of a two generation family. The translocation was observed in the father, one of the sons (aged 10 years when included in the study), and the daughter (newborn) (Fig. 1). The father and the son carrying the translocation had LNN, with >150 nevi. In addition, the son with the translocation had lentigines on the face, one café-au-lait macule and despite strictly avoiding exposure to sunlight developed an in situ CMM at the age of 16 years (Fig. 1). The daughter developed almost 20 nevi at the age of 5 years, being phototype IV (dark skin color), while the father and the affected son had phototype II (light skin color with difficulties to tan and easily sunburned). The nevi present in individuals with the translocation were macular lesions, dark in color with atypical reticulated pattern under dermoscopy, suggestive of lentiginous junctional or compound atypical nevi. The mother had a few moles of a globular pattern under dermoscopy, suggestive of compound/dermal nevi, and multiple lentigines and other signs of photo-aging. The son without the translocation had fewer than 50 moles at the age of 14 years, most of which were dermal or compound type, being phototype II.

FISH analysis using genomic clones localized the translocation breakpoints to within RP1-121K10 and between RP11-92311 and RP11-1100L3 clones at p921 and q12, respectively (Supplementary Fig. 1; Supplementary material for this article can be found at http://www.interscience.wiley.com/
jpages/1045-2257/suppmat). Thus, the breakpoints were primarily mapped to between the TEK and C9orf14 genes, and between the ANKRD33 and ACVRL1 genes at 9p21 and 12q13, respectively.

To focus the study on the translocation breakpoint that could potentially be the cause of LNN and subsequently CMM, we examined whether genomic regions on 9p21 or 12q13 are lost in melanoma. For this analysis, a publicly available data set of genome-wide DNA copy number alterations in 126 primary melanomas was used (Curtin et al., 2005). The results showed no alterations of 12q13 but common loss of 9p21 including TEK and C9orf14 loci (Fig. 2A). A total of 30 out of 126 (23%) tumors showed relative loss of this region located proximal to the CDKN2A gene. CDKN2A is a tumor-suppressor gene mutated in melanoma (Serrano et al., 1996).

Different types of genetic and molecular evidence suggest the existence of an additional tumor-suppressor at 9p21. Mutational analysis of melanoma-prone families with linkage to 9p21 did not identify alterations in CDKN2A, CDKN2B, or p14ARF in a significant proportion of cases (Liu et al., 1997; Laud et al., 2006). Loss of heterozygosity of a region proximal to the CDKN2A locus has been described in CMM and lung carcinoma tumors (Puig et al., 1995; Kim et al., 2006). Loss of heterozygosity at 9p21 was described in melanoma (van't Veld, 1999). Functional chromosome transfer studies identified distinct regions at 9p21 implicated in the development of CMM (Parris et al., 1999). Finally, a locus modifying CMM risk located proximal to CDKN2A was described in melanoma families harboring CDKN2A mutations (van der Velden et al., 1999)

FISH analysis and Southern blotting excluded the disruption of TEK and C9orf14 genes by the translocation breakpoint (not shown). The breakpoint was then finely mapped by designing Southern probes at regular intervals between TEK and C9orf14 using information taken from the human genome sequence (PCR primers for probes are detailed in Supplementary Table 1; Supplementary material for this article can be found at http://www.interscience.wiley.com/jpages/1045-2257/suppmat). The breakpoint was limited to a 1.5 kb region, at ~33 kb proximal and ~20 kb distal from 3'-TEK and 5'-C9orf14 genes, respectively (Fig. 3).

The level of sequence conservation across genomes of different species is relatively high around the breakpoint, which suggests the presence of conserved regulatory or expressed sequences between TEK and C9orf14 genes.

To determine the existence of an unidentified gene between TEK and C9orf14, three complementary molecular approaches were used: (i) complete sequencing of public domain EST clones; (ii) normal human melanocyte cDNA library screening; and (iii) RT-PCR reactions using cDNA preparations from different tissues (including normal skin tissue), cell types (including normal human epidermal melanocyte cell cultures) and primers designed in evolutionarily conserved sequence regions (Supplementary Table 1). First, complete sequence of all EST clones in the region was obtained, with an exon–intron structure found in only one of them (IMAGE 2275835). This clone and the overlapping clone IMAGE 1677562 have polyA+ sequences that define a full transcript of ~1.5 kb in length. A possible ORF within this transcript encodes for a protein of 99 amino acids (AA).

Subsequently, melanocyte cDNA library screening using probes belonging to all ESTs identified a single polyA+ clone that corresponded exactly to the 1677562 sequence. Northern analysis of different tissues using this sequence as a probe revealed a complex expression pattern consisting of at least six different transcripts of up to ~6 kb, and main expressed transcripts of ~0.5 and ~1.5 kb (Supplementary Fig. 2; Supplementary material for this article can be found at http://www.interscience.wiley.com/jpages/1045-2257/suppmat), the latter in agreement with the expected mRNA size based on sequencing of ESTs.

Finally, RT-PCR reactions extended the cDNA sequence distally from clones 2275835 and 1677562. Thus, 13 additional exons were identified. On the other hand, 5'-RACE reactions extended the cDNA proximally, 208 bp to the 5' end of 2275835 sequences. It was therefore determined that the complete C9orf14 gene sequence contains 17 exons and that the translocation breakpoint is located in the 10th intron. Searching for additional functional elements at the C9orf14 locus, we identified a small noncoding RNA complementary to the mRNA sequence of C9orf14 represented by the first two exons, suggesting complex regulation of C9orf14 expression (Supplementary Fig. 2).

It was then analyzed whether C9orf14 is differentially expressed between cancer cell lines and between melanoma tumors relative to nevi and normal melanocytes. Using a publicly available expression data set containing 60 cancer cell lines of different tissue origin (NCI60) (Scherf et al., 2000), differential expression of the AI078569 probe corresponding to the 1677562 sequence was observed (Fig. 2B). Thus, three (SK-MEL-5 and 28, and MALME-3M) out of seven melanoma cell lines showed downregulation of C9orf14. No altera-
Figure 2. DNA copy number alteration and gene expression analysis of 9p21 and 12q13 loci. A: Clustering of melanoma tumors based on DNA copy number analysis (log2 tumor/normal tissue ratios) of complete human chromosomes 9 (HSA9) and 12 (HSA12). Sample and clone identifications are not shown because of resolution constraints [available in GEO record GSE2631 (Curtin et al., 2005)]. Common loss of 9p21 including $\text{CDKN2A}$ and $\text{TEK-C9orf11}$ loci is observed. No alterations of HSA12 are observed except for low-frequency amplification that includes $\text{ACVR1L}$ and $\text{ACVR1B}$ loci. B: NCI60 cancer cell lines gene expression analysis at 9p21 and 12q13 loci. Melanoma cell lines are shown in red. Differential expression (white squares) between melanoma cell lines is shown for $\text{AI078569}$ (IMAGE 1677562 sequence; $\text{C9orf14}$). Two probes match the $\text{CDKN2A}$ gene in this U95 platform: 73130_s_at (U26727) and 73132_r_at (AA909181). As expected, 73130_s_at probe indicates downregulation of $\text{CDKN2A}$ in most melanoma cell lines. However, 73132_r_at probe does not correlate with the expected results. This apparent inconsistency is probably due to the Affymetrix heuristics rules being relaxed (r-suffix) for this particular probe, which suggests poorer reliability. No comparable expression differences between melanoma cell lines are observed for 12q13 probes. C: $\text{C9orf14}$ expression levels (log2 transformed) in normal skin, melanocytes, keratinocytes, nevi, and melanomas. Results are shown for Affymetrix U133 Plus 2.0 probe 1563728_at. Downregulation is particularly observed in late stage melanomas.
tions were observed in probes located on 12q13. These observations could suggest genetic or epigenetic alterations of the C9orf14 locus associated with CMM development. Analysis of an expression data set (GEO record GSE4587) (Smith et al., 2005) containing data from normal skin, short-term cultures of epidermal melanocytes and keratinocytes, nevi and melanomas, revealed downregulation of C9orf14 in melanomas (P < 0.05) (Fig. 2C). Specifically, downregulation was observed in late stage melanomas (vertical growth phase, metastatic growth phase, and lymph node metastasis).

Downregulation of C9orf14 expression in ovarian cancer cell lines was also observed in the NCI60 set (OVCAR-5 and 8, and SK-OV-3). Analysis of a publicly available ovarian expression data set (Lu et al., 2004) revealed downregulation of C9orf14 in serous carcinomas relative to normal ovarian surface epithelium (P < 0.05). This observation, together with data describing the genetic loss of 9p21 in ovarian clear cell carcinomas (Dent et al., 2003), might also suggest that C9orf14 is involved in ovarian tumorigenesis.

Next, the role of the C9orf14 encoded protein of 99 AA was investigated through an analysis of its cellular localization in normal human mammary epithelial cells (HME/TERT) (DiRenzo et al., 2002). Centrosome localization was then revealed by colocalization of EmGFP-C9orf14 and γ-tubulin (Fig. 4). The centrosome localization could suggest a role in micro
tubule organization that influences metastatic potential and chromosome segregation fidelity (Doxsey et al., 2005). A critical role for the CDKN2A gene product preventing centrosome dysfunction in primary cells has recently been reported (McDermott et al., 2006) and may point to a functional association with the \textit{C9orf14} gene product.

Taken together, the molecular characterization of a chromosome translocation in related individuals with LNN and CMM, and integrative genomics analysis suggests that \textit{C9orf14} is a tumor-suppressor for nevus development, late stage melanoma, and, maybe, for other types of human cancers. The strategy developed here can be applied to other genes in which an underlying molecular mechanism is suspected. Thus, the integration of “omic” data highlights likely candidates with increased confidence (Mootha et al., 2003; Dahia et al., 2005). Genetic analysis of melanoma-prone families with linkage to 9p21 but without alterations in CDKN2A, CDKN2B, or p14ARF, together with population-based association studies, should define the role of \textit{C9orf14} in melanoma susceptibility.

\textbf{REFERENCES}


