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Molecular Characterization of Human Cutaneous Melanoma-derived Cell Lines

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Abstract. *Background: Several studies have demonstrated that different genetic profiles contribute to melanoma development and progression. Materials and Methods: To evaluate the existence of different molecular aberration patterns in melanoma associated with v-raf murine sarcoma viral oncogene homolog B1 (BRAF) or 9p21 locus alterations, eleven patient-derived melanoma cell lines were characterized. Multiplex ligation probe amplification (MLPA) was used to detect chromosomal alterations. Single- strand conformation analysis and sequencing were performed to study BRAF, neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (c-KIT), melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor) (MC1R), cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4) genes. Results: BRAFV600E mutation was detected in 54% of cell lines. NRAS was mutated in one cell line also carrying multiple copies of NRAS. All cell lines with MC1R variants harboured BRAFV600E. Concurrent loss of MUTYH (1p33), gains of c-MYC (8q24) and of CDK6 (7q21) were found to be significantly associated in cell lines (45%) that harboured biallelic 9p21 deletions including CDKN2B-CDKN2A-MTAP. Conclusion: These data suggest the existence of a specific pattern of somatic alterations in genes that are involved in DNA repair (MUTYH) and in cell cycle regulation (c-MYC, CDK6, CDKN2A and CDKN2B). Interestingly, all MC1R variants were associated with BRAFV600E and all cell lines from visceral metastases harboured BRAFV600E.*

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Melanoma is a complex and heterogeneous disease. Several studies have identified loci with established importance (1). The 9p21 region has been widely studied since deletions affecting this region have been reported in approximately 50% of tumours, being higher in cultured cell lines (2, 3). The 9p21 locus contains a well known tumour- suppressor gene (TSG), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), which encodes two distinct cell cycle regulatory proteins: $p16^{INK4A}$ and $p14^{ARF}$. Comparative microarray analysis in melanoma cell lines with and without homozygous deletion of *CDKN2A* showed several classes of genes involved in different pathways (4). Loss of heterozygosity (LOH) studies in melanoma tumours suggest the existence of other TSGs located at 9p21 (5).

Greshock *et al*. (6) have shown that DNA copy number aberrations associated with v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*)-mutated melanomas are different from those found in neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*)-mutated and *BRAF*/*NRAS* wild-type tumours. Previously, two expression studies revealed a *BRAF* mutation-associated expression signature, finding a number of significant differentially expressed genes comparing melanoma cell lines with and without *BRAF* mutations (7, 8). However, another study concluded that there was no specific gene expression profile associated with *BRAF* mutation status (9).

The main objective of this study was to investigate the existence of different molecular aberration patterns associated with BRAF- activating mutations and 9p21 alterations in a set of eleven human cutaneous melanoma-derived cell lines by Multiplex ligation probe amplification (MLPA) approach. Furthermore, *BRAF*, *CDKN2A*, cyclin-dependent kinase 4 (*CDK4*), v-kit Hardy-Zuckerman 4 feline sarcoma viral

oncogene homolog (*c-KIT*), melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor) (*MC1R*) and *NRAS* genes were also studied by mutational analysis.

Materials and Methods

Cell lines and culture conditions. Eleven human malignant melanoma cell lines were obtained from Caucasian patients with sporadic melanoma (10). Melanoma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Barcelona, Spain), supplemented with 10% foetal calf serum (FCS) (Gibco) and antibiotics (penicillin/streptomycin; Sigma/Aldrich, Madrid, Spain). All the cells were incubated at 37˚C in an atmosphere with 5%-CO_2.

DNA extraction. The PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) was used to isolate genomic DNA from cell lines according to the manufacturer's instructions.

PCR amplification. Promoter (-34G>T variant), intronic (IVS2-105) and coding regions of the CDKN2A gene (exons 1α, 2 and 3 of $p16^{INK4A}$ and exon 1β of $p14^{ARF}$) and exon 2 of *CDK4*, *MC1R* and *c-KIT* exons 9, 11, 13, 17, 18 were amplified by PCR using primers and conditions previously described (11-14). For *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2, primers were designed to amplify the exons where the most common mutations are detected. All PCRs were carried out using the PCR Master Mix (Promega Co., Madison, WI, USA) following the manufacturer's instructions. PCR conditions were: initial denaturizing step at 95˚C for 5 min, followed by 35 cycles (95˚C for 1 min, Tm (*c-KIT* 56˚C, *BRAF* 56˚C, *NRAS* 57˚C and *MC1R* 55˚C) for 1 min, 72˚C for 1 min), and a final extension at 72˚C for 10 min and maintaining at 4˚C until single- strand conformational polymorphism (SSCP) or sequencing studies were carried out. Primers for *BRAF* exon 11F: TTTCTTTTTCTGTTTGGCTTG, 11R: TGTGGTGACATT GTGA CAAGT, exon 15F: TGCTTGCTCTGATAGGAAAA, and exon 15R: TGAGGCTATTTTTCCACTGA; for *NRAS* exon 1F: CGCCAATTAACCCTGATTAC, exon 1R: GCTGACCTGATCCT GTCTCT, and exon 2F: CCCCTTACCCTCCACACC, exon 2R: TCTGAAAGGATGATCTTTGTGTT.

Mutational analysis. Mutation screening for *BRAF*, *CDK4*, *CDKN2A*, *c-KIT* and *NRAS* loci was performed by SSCP (15). Samples with abnormal migration products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in an ABI3100 automatic sequencer (Applied Biosystems). *MC1R* was directly sequenced. Specific internal *MC1R* primers were designed to analyze the entire coding sequence (INT-F: TACATCTCCATCTTCTACGC and INT-R: GTGCTGAAGACGACACTG).

MLPA. Copy number variations were analyzed by MLPA (P024B CDKN2A/2B, P172 and P027) (MRC-Holland, Amsterdam, Netherlands). All kits were used according to the manufacturer's instructions. Amplified samples were analysed on automated sequencer (Applied Biosystems). MLPA results were evaluated using a custom MLPA analysis programme (SeqPilot- JSI Medisys, Kippenheim, Germany). For each fragment, the peak area was calculated and normalized against the mean peak area of control samples (consisting of six human DNA from normal tissue with normal gene dosage). A difference was considered significant if the ratio was less than 0.5 (loss) or higher than 1.5 (gain). A ratio close to 1.0 indicates two copies present (*i.e.* wild-type, wt); 0.00, both copies deleted (*i.e.* homozygous deletion, HD); 0.5, one copy deleted (*i.e.* loss of heterozygosity, LOH), 1.5 or higher, one copy duplicated or multiple copies. Experiments were carried out in duplicate. For each cell line, results from the MLPA kits employed were combined and arranged in figures to represent chromosome order.

Statistical analysis. Fisher's exact test (two-sided) was used to evaluate correlations between copy number alterations. P-values <0.05 were considered significant.

Results

Analysis of BRAF, NRAS, c-KIT, MC1R and CDK4 mutational status. Eleven cultured melanoma cell lines were analysed for mutations in exons 11 and 15 of the *BRAF* gene, and exons 1 and 2 of the *NRAS* gene, using SSCP and sequencing. Six out of eleven cell lines carried activating mutations in *BRAF* (BRAFV600E). The mutation involving the hotspot codon 600 in exon 15 was c.1799T>A in all cell lines except in one (CPM) carried c.1798-99GT>AA tandem mutation causing, in all cases, a valine-to-lysine amino acid change at residue 600 (Table I). Interestingly, all cell lines derived from visceral metastases belonged to the *BRAF*V600E carrying cell lines, while all cell lines from cutaneous metastases except one belonged to the *BRAF* wild-type (*BRAF*wt) (Table I).

Somatic mutation affecting *NRAS* was detected in one cell line (TPR), which carried a missense mutation changing a glutamine to histidine at hotspot codon 61 (p.Q61H, c.183A>C) (Table I). No mutations were detected in c-*KIT*. Three c-*KIT* variants were found in 45% of the cell lines, all of them with *BRAF* or *NRAS* mutated (Table I). M36 carried p.I798I (c.2394C>T) in exon 17. The variant p.L862L (c.2586G>C) in exon 18 was detected in cell lines TPR, DB, CPM and M16, which also carried IVS16-77G>A in intron 16. All the variants have previously been described except IVS16-77G>A.

DNA sequence analysis of the *MC1R* gene revealed that four out of sixof the *BRAF*V600E cell lines carried concomitant *MC1R* allelic variants (Table I). The M3 cell line had variants p.R142H (c.425G>A) and p.V60L (c.178 G>T). Cell line M16 harbored variant p.R163Q (c.488G>A), and p.T314T (c.942A>G) and p.V92M (c.274 G>A) variants were present in the DB cell line. Cell line JC carried a p.T272M (c.815C>T) change. All the variants have previously been described. In contrast, *MC1R* variants were not detected in the *BRAF*wt cell lines.

SSCP analysis of exon 2 of the *CDK4* gene, and DNA sequencing revealed no mutations in any of the cell lines.

Analysis of copy number alterations. The presence of deletions affecting the 9p21 region was evaluated by MLPA assay. Overall, almost all the cell lines (10/11) carried deletions within the *CDKN2A-CDKN2B-MTAP* region (Table II). However, retention of cyclin-dependent kinase inhibitor 2B (*CDKN2B*) and of methylthioadenosine phosphorylase (*MTAP*) was found

Table I. *Mutational Analysis of BRAF, NRAS, MC1R, c-KIT and CDK4 genes.*

Cell line	Origin	Primary tumor	BRAF Exon 15	NRAS Exon 2	MC1R	c-KIT exons 17-18	CDK4 Exon 2
M3	ViscM	*Nd*	**V600E (c. 1799T>A)**	WT	**R142H (c.425G>A), V60L (c.178G>T)**	WT	WT
JC	ViscM	**NM**	**V600E (c. 1799T>A)**	WT	**T272M (c.815T>C)**	WT	WT
DB	ViscM	*Nd*	**V600E (c. 1799T>A)**	WT	**T314T (c.942A>G), V92M (c.274G>A)**	IVS16-77G>A; L862L(c.2586G>C)	WT
M36	ViscM	*Nd*	**V600E (c. 1799T>A)**	WT	WT	I798I(c.2394C>T)	WT
CPM	Nd	*Nd*	**V600E (c.1798-99GT>AA)**	WT	WT	IVS16-77G>A; L862L(c.2586G>C)	WT
M16	CM	**NM**	**V600E (c. 1799T>A)**	WT	**R163Q (c.488G>A)**	IVS16-77G>A	WT
M17	CM	**SSM**	WT	WT	WT	WT	WT
M28	CM	**ALM**	WT	WT	WT	ND	WT
GE	CM	*Nd*	WT	WT	WT	WT	WT
TPR	CM	**NM**	WT	**Q61H (c.183A>C)**	WT	IVS16-77G>A; L862L(c.2586G>C)	WT
M9	PT	**NM**	WT	WT	WT	WT	WT

Mutations are shown in bold. Nd: No data. CM: Cutaneous metastasis; PT: primary tumor; ViscM: visceral metastasis; WT: wild type. NM: Nodular melanoma; SSM: superficial spreading melanoma, ALM: acral lentiginous melanoma. *BRAF* exon 15 and 11, *NRAS* exon 1 and 2, c-*KIT* exons 9, 11, 13, 17 and 18, *CDK4* exon 2 were analyzed. The entire coding region of *MC1R* was analyzed.

in two and five cases, respectively. Homozygous deletions were present in 72% of cell lines (8/11), while loss of heterozygosity (LOH) was observed in 18% (2/11). Only the DB cell line retained all loci. Furthermore, three samples harboured hemizygotic deletions. In cell line M36, the affected loci were *CDKN2A-CDKN2B*. In cell line M16 LOH involved tyrosine kinase, endothelial (*TEK*), embryonic lethal, abnormal vision, Drosophila-like 2 (*ELAVL2*) and also part of *MTAP* to the interferon, beta 1, fibroblast (*IFNB1*) gene. TPR presented hemizygotic deletions of practically the whole 9p21 region. A complex pattern was observed in the M28 cell line including homozygous deletions *CDKN2A-CDKN2B* and partial deletions of *MTAP*, as well as of the IFNB1 gene. Only the CPM cell line was affected by an amplification encompassing the region from kelch-like 9 (Drosophila) (*KLHL9*) to the myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3 (*MLLT3*) gene. Homozygous deletion in melanoma cell lines was confirmed by PCR amplification of *CDKN2A*. In only three cell lines *CDKN2A* was amplified completely: M36, TPR and DB. The absence of amplification correlates with the homozygotic deletions detected with MLPA. Sequencing of PCR products revealed the presence of CDKN2A point mutations in two cases. Whereas the TPR cell line presented a proline-to-leucine substitution at codon 114 (c.341CC>TT) in the retained allele, the DB cell line carried nonsense mutation p.R58X (c.172 CC>TT) in homozygosis.

To further characterize the cell lines, additional MLPA probes were used to enable the analysis of DNA copy number losses and gains in chromosomal regions known to be relevant in melanoma pathogenesis. We identified a significant correlation between losses of mutY homolog (*E. coli*) (*MUTYH*) (1p33), gains of v-myc myelocytomatosis viral oncogene homolog (avian) (c-*MYC*) (8q24) and gains of cyclin-dependent kinase 6 (*CDK6*) (7q21.3) ($p<0.05$) in cell lines (45%) that harboured homozygous deletions in 9p21 restricted to *CDKN2A* and *CDKN2B* or *MTAP* but not affecting contiguous genes in the 9p21 region. 9p21 deletions in the *CDKN2A* region found by MLPA were confirmed by mutational analysis of *CDKN2A* (Table II).

No other chromosomal alteration was found to be associated with either BRAF or the 9p21 locus.

Discussion

In the present study eleven human cutaneous melanoma cell lines were genetically characterized for the presence of genetic aberration patterns.

The MAPK pathway is a key regulator of melanoma cell proliferation, as dysregulation of this pathway has been identified by gain of function mutations in *NRAS* or *BRAF* in approximately 13% and 60% of melanoma cell lines (2). In our study, 54% of melanoma cell lines (6/11) carried *BRAF* mutations. *NRAS* mutation was detected in one cell line with *BRAF*wt (p.Q61H mutation). As previously reported, we found a strong association between *MC1R* variants and *BRAF* mutations (16). It is believed that increased generation of reactive oxygen species in carriers of *MC1R* variants induces the A>T transversion characteristic of the common p.V600E *BRAF* mutation (16). We did not identify a genomic profile related to *BRAF* mutations.

Table II. *Summary of Multiplex ligation probe amplification (MLPA) results*.

Gene Symbol	Gene Name	Position	M3	JC	M9	M17	GE	M36	CPM	M16	TPR	M28	DB
TEK	tyrosine kinase, endothelial	9p21.2							■	▒			
ELAVL2	embryonic lethal, abnormal vision, Drosophila)-like 2	9p21.3							▒	▒	▒		
CDKN2B	cyclin-dependent kinase inhibitor 2B	9p21.3	■	■	■	■		▒	■	■	▒	■	
CDKN2A	cyclin-dependent kinase inhibitor 2A	9p21.3	■	■	■	■	■	▒	■	■	▒	■	
MTAP	methylthioadenosine phosphorylase	9p21.3		■			■		■	▒	▒	▒	
*KLHL9**	Kelch-like 9 (Drosophila)	9p21.3							♦	▒	▒		
IFNW1	interferon, omega 1	9p21.3							♦	▒	▒		
IFNB1	interferon, beta 1, fibroblast	9p21.3							♦	▒	▒	▒	
MLLT3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3	9p21.3							♦		▒		
MFN2	mitofusin 2	1p36.22	▒	▒			▒		▒			■	
NBL1	neuroblastoma, suppression of tumorigenicity 1	1p36.13		▒								■	
PTAFR	platelet-activating factor receptor	1p35.3	▒	■			▒		▒			■	
MYCBP	c-myc binding protein	1p34.2		▒								■	
MUTYH	mutY homolog (E. coli)	**1p33**	▒	▒	▒	▒	▒						
RPE65	retinal pigment epithelium-specific protein 65kDa	1p31.2		▒			▒	▒	▒			■	
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1p13.2									♦		
CDK6	cyclin-dependent kinase 6	**7q21.3**	♦	♦	♦	♦	♦	♦					
c-MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	**8q24.12**	♦	♦	♦	♦	♦						
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	11q13.1					♦			♦			
GSTP1	glutathione S-transferase pi 1	11q13.2					♦			♦			
CCND1	cyclin D1	11q13.3					♦			♦			
EMS1	cortactin	11q13.3					♦			♦			
FGF3	fibroblast growth factor 3	11q13.3					♦			♦			
BIRC3	baculoviral IAP repeat containing 3	11q22					♦			♦			
CCND2	cyclin D2	12p13.32		♦			♦				♦	♦	♦
BCLG	BCL2-like 14	12p13.2		♦								♦	
BCL2L1	BCL2-like 1	20q11.1			♦								♦
PTPN1	protein tyrosine phosphatase, non-receptor type 1	20q13.1			♦			♦	♦				♦
AURKA	aurora kinase A	20q13.31			♦			♦	♦			♦	♦
UCKL4	uridine-cytidine kinase 1-like 1	20q13.33			♦			♦	♦				♦

The locations of MLPA probes are indicated by gene name and chromosomal position. All cell lines, except DB, present deletions of 9p21 region. Biallelic deletions of 9p21 region including only *CDKN2A*, *CDKN2B* and *MTAP* genes were present only in cell lines carryng MUTYH/c-MYC/CDK6 alterations. Black square: homozygous deletion; grey square: loss of heterozygosity; diamonds♦: duplication. **KLHL9* was previously named *KIAA1354*.

A specific pattern of copy number variation losses of *MUTYH* and gains of c-*MYC* were significantly associated with gains of *CDK6*.

Frequent LOH on chromosome 1p indicates the existence of putative TSGs in this region that may predispose to tumor development or contribute to tumor progression.

The MUTYH protein is a base excision repair enzyme involved in repair of DNA damage caused by oxidative stress. Defects in this repair mechanism leaded to accumulation of mutations (17). Recently, Santonocito *et al*., has shown that *MUTYH* germline variants Y165C, G382D and V479F, known to be associated with adenomatous polyposis and colorectal cancer, are not associated with melanoma risk (18, 19). This finding is in contrast with ours. One explanation for these discrepant results is that *MUTYH* variants are located in exons 7, 13 and 15 whereas the MLPA probe for *MUTYH* gene hybridized to a region of exon 5. Furthermore, two putative melanoma susceptibility loci on chromosome 1p (1p22, 1p36) have been identified (20-23). To date, no candidate genes have been identified at either 1p22 or 1p36. Although not previously associated with melanoma, due its function and location, *MUTYH* could be a candidate melanoma gene.

c-MYC is a cell-cycle regulator that influences the activity of CDK4/CDK6 complex, which is critical for G_1 progression and G_1/S transition (24). Overexpression of c-MYC has been found in 40% of melanomas (25). Zhuang *et al*. (26) found that one of the major functions of c-MYC overexpression during melanoma progression is to continuously suppress senescence induced by mutated BRAF or NRAS. Regarding *CDK6*, large genomic amplifications of 7q21 in 59% of melanoma cell lines have been reported (27).

Losses of *MUTYH* and gains of *c-MYC* and *CDK6* genes were found among cell lines carrying biallelic deletions of the 9p21 region including *CDKN2A* and *CDKN2B* or *MTAP* genes. Deletions associated with the loss of *TSGs CDKN2A* and *CDKN2B* have been frequently found in melanoma cell lines (4) and impaired the production of $p15^{INK4B}$, $p16^{INK4A}$ and $p14^{ARF}$ proteins. Biallelic deletions in *CDKN2A* are associated with reduced overall survival (28). Given that, the *MUTYH-MYC-CDK6* pattern is expected to be involved in tumor progression rather than initiation. *MTAP* gene is located approximately 100 kb telomeric of *CDKN2A* and therefore may also serve as a TSG. The loss of MTAP expression has been detected in melanoma cell lines and had an effect on tumour progression (29).

Other alterations in the 9p21 locus involving point mutations, large deletions and duplications were observed in cell lines without loss of *MUTYH* and gains of c-*MYC* (Table II). CDKN2A point mutations were detected in two cases. The TPR cell line showed hemizygotic deletions of the whole 9p21 region and carried a missense mutation (p.P114L) in the retained allele. The $p16^{INK4A}$ protein with this missense mutation loses the ability to bind CDK4/6 complexes (30). The DB cell line presented a normal DNA copy number 9p21 locus and a premature *CDKN2A* stop codon (p.R58X) in homozygosis, leading to production of non-functional $p14^{ARF}$ and $p16^{INK4A}$. The possible mechanism leading to this homozygotic mutation would be loss of the chromosome harbouring the wild-type allele followed by chromosomal duplication or gene conversion on the other allele. Both mutations detected in *CDKN2A* are UV signature mutations characterized by C-T and CC-TT transitions at the dipyrimidine site, suggesting the probable UV aetiology of the mutations (31).

There is evidence that there is more than one TSG situated in the 9p21 region (5). Hemizygous deletions of tumour-suppressor *IFNB1* and *IFNW1* indicated that defects in the IFN gene cluster could act as a selective event for growth advantage. Surprisingly, in the CPM cell line, we detected duplication, including *KIAA1354*, *IFNW1*, *IFNB1* and *MLLT3* genes, to the homozygously deleted region. This amplification could be due to chromosomal copy number alterations

This study provides a comprehensive characterization of eleven melanoma cell lines using mutational analyses and MLPA. This approach offers a considerable advantage over other established techniques due to its low cost, the small quantities of DNA required and the possibility to test multiple loci in a single reaction. Limitations of this study include the small sample size. Despite the low number of samples, it was possible to confirm known mutations and their frequencies in human melanoma, such as *BRAF*V600E and *NRAS*.

Conclusion

In conclusion, a specific pattern of somatic alterations was described in genes that are involved in DNA repair (*MUTYH*) and in cell cycle regulation (c-*MYC*, *CDK6*, *CDKN2A* and *CDKN2B*). *MC1R* variants were associated with the *BRAF*V600E carrying group of melanoma cell lines. Among known genes implicated in melanoma, our study suggests the involvement of *MUTYH* in melanoma development. Further studies with larger numbers of samples are imperative as the role of *MUTYH* in melanoma needs to be clarified. Interestingly all the melanoma cell lines derived from visceral metastases belong to the *BRAF*V600E carrying group, while all cell lines from cutaneous metastases except one belong to the *BRAF*wt group.

Conflicts of Interest

All authors declare no conflicts of interest or financial disclosures.

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