

The Comparison of Hotspot Mutations in Primary and Metastatic Melanoma Cell Lines

Primer ve Metastatik Melanom Hücre Hatlarında Sıcak Nokta Mutasyonlarının Karşılaştırılması

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Abstract

Objective: Melanoma is a disease that develops as a result of UV radiation acting as a mutagenic risk factor and causes various genetic changes. In our study, we aimed to determine the changes in human cancer-related genes between Primary Melanoma (WM115) and Metastatic Melanoma (SK-MEL-30) cell lines.

Materials and Methods: Among the proto-oncogenes and tumor suppressor genes, 50 genes associated with human cancers were selected. Afterwards, the changes of hotspot mutations occurring in 50 genes between the two cell lines were examined using the Ion Ampliseq Cancer Hotspot DNA Panel v2 gene panel.

Results: A mutation increase was observed in the metastatic melanoma cell line compared to the primary cell line. 4 Variant of Uncertain Significance (VUS), 3 benign and 2 pathogenic mutations were detected in the WM115 cell line. Pathogenic mutations have been identified in the BRAF proto-oncogene. In the SK-MEL-30 cell line, 24 VUS, 1 benign, and 1 possible pathogenic mutation (in the VHL gene) were observed.

Conclusions: According to the results of our research, we think that the occurrence of BRAF mutations in primary melanoma indicates that this gene is effective in the progression and development of melanoma, and the mutation occurring in the VHL gene of metastatic melanoma might be effective in carcinogenesis via the HIF pathway. In addition, most of the VUS mutations of metastatic melanoma occurred in the RET and NOTCH pathways. The accumulation of mutations in genes involved in these pathways may contribute to the development of metastasis.

Keywords: Hotspot mutation, melanoma, next generation sequencing, NOTCH, RET

Öz

Amaç: Melanom, UV radyasyonunun mutajenik bir risk faktörü olarak hareket etmesi sonucu gelişen ve çeşitli genetik değişikliklere neden olan bir hastalıktır. Çalışmamızda Primer Melanom (WM115) ve Metastatik Melanom (SK-MEL-30) hücre hatları arasında insan kanser ilişkili genlerindeki değişiklikleri belirlemeyi amaçladık.

Materyal ve Metot: Proto-onkogenler ve tümör baskılayıcı genler arasından insan kanserleriyle ilişkili 50 gen seçildi. Daha sonra iki hücre hattı arasında 50 gende oluşan hotspot mutasyonlarının değişimleri Ion Ampliseq Cancer Hotspot DNA Panel v2 gen paneli kullanılarak incelendi.

Bulgular: Metastatik melanom hücre hattında primer hücre hattına göre mutasyon artışı gözlemlendi. WM115 hücre hattında 4 adet Önemi Belirsiz Varyant (VUS), 3 adet benign ve 2 adet patojenik mutasyon tespit edildi. BRAF proto-onkogeninde patojenik mutasyonlar tanımlanmıştır. SK-MEL-30 hücre hattında 24 VUS, 1 iyi huylu ve 1 olası patojenik mutasyon (VHL geninde) gözlemlendi.

Sonuç: Araştırmamızın sonuçlarına göre, primer melanomda BRAF mutasyonunun görülmesinin, bu genin melanomun ilerlemesi ve gelişiminde etkili olduğunu, metastatik melanomda ise VHL geninde oluşan mutasyonun ise HIF yolu ile karsinogeneze etkili olabileceğini düşünmekteyiz. Ek olarak, metastatik melanomun VUS mutasyonlarının çoğu RET ve NOTCH yollarında meydana geldi. Bu yollarda yer alan genlerdeki mutasyonların birikmesi, metastaz gelişimine katkıda bulunabilir.

Anahtar Kelimeler: Hotspot mutasyonu, melanom, NOTCH, RET, yeni nesil dizileme

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INTRODUCTION

Melanoma is caused by the uncontrolled proliferation of melanocytes in the basal layer of the epidermis.¹ The primary cause of melanoma is largely due to environmental factors, particularly exposure to UV radiation, with genetic predisposition being a less common contributor.² Most of the changes underlying the genetic basis of melanoma occur as randomly acquired mutations within melanocytes.³

Tumor genomes can contain tens of thousands of somatic mutations. Somatic mutations in cancer genomes are classified as driver and passenger according to their functional consequences. It is also known that somatic mutations in cancer genomes are often repeatedly observed at specific amino acid residues (hotspot points). A recent comprehensive analysis of the Cancer Genome Atlas PanCancer (TCGA PanCan) project identified approximately 500 single amino acid-level mutational hotspots in well-known and novel cancer-associated genes.⁴ Studies have shown that genetic alterations include gain-on-function of proto-oncogenes and loss-of-function of function on tumor suppressor genes.⁵ The activation mechanisms of proto-oncogenes are as follows: chromosomal translocation of a proto-oncogene from a non-transcribable location to an adjacent transcribable location, point mutation resulting from the substitution of a single base by another base, gene amplification, and inclusion of a transcription-inducing gene near the proto-oncogene.⁶

Tumor suppressor genes (TSGs) can be classified into cellular signalling functions such as cell growth and proliferation, DNA repair mechanisms and the induction of apoptosis. TSGs have three important properties: 1) they are recessive at the cellular level and both alleles are typically inactivated in tumors, 2) the inheritance of a single mutant allele increases tumor susceptibility, and 3) the same gene is frequently inactivated in sporadic cancers.⁷ In melanoma, both proto-oncogenes and TSGs are important topics of recent research.⁸

We aimed to characterize the precursor mutations that occur with the progression of Melanoma by detecting alterations in human cancer-associated genes between WM115 and SK-MEL-30 cell lines. We hypothesized that the numerical and pathway-based differences of hotspot mutations observed between primary and metastatic melanoma cell lines might be decisive in explaining molecular mechanisms of melanoma progression.

MATERIALS AND METHODS

Ethics Committee Approval: Ethical approval for the study is not required. In this study, secondary cell culture was used.

Cell Lines: Human primary melanoma cell line (WM115) (WM115-01-0001) was obtained from Rockland company, and human metastatic melanoma cell line (SK-MEL-30) (03010901) was obtained from the Foot and Mouth Institute Cell Culture Bank of the Ministry of Agriculture and Forestry of the Republic of Türkiye. Cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) (Capricorn Scientific, DMEM-HPA) containing 10% Fetal Bovine Serum (FBS) (Capricorn Scientific, FBS-HI-11A), 1% Penicillin/Streptomycin antibiotic (P/S) (Capricorn Scientific, PS-B) and 1% L-Glutamine.

Cell Lysate Preparation: The cells were removed from plates and transferred to the falcon tube. It was centrifuged at 200 g for 5 min, and the supernatant was discarded. Fresh medium was placed in the tube was vortexed. After that, it was kept at -80°C for 3-5 minutes, then taken to 25°C. It vortexed after thawing, then centrifuged again at 200 g for 5 minutes. This process was repeated 3 times (-80°C /25 °C/ Centrifuge).

DNA Isolation: DNA isolation was performed for the Invitrogen PureLink® Genomic DNA Mini Kit (K1820-01).

In the first step, 200 µl of the cell lysate sample was suspended with 200 µl of PBS in a 2 ml Eppendorf tube. 20 µl proteinase K and 20 µl RNAase were added and vortexed briefly. After incubating at 25 °C for 2 min, 200µl lysis/binding buffer was added and vortexed (20-30 s) until a homogenous solution was obtained. The solution was incubated at 55°C for 10 min. After incubation, 200 µl of 96-100% ethyl alcohol was added to the lysate and vortexed for 5 seconds. The lysate was transferred to spin columns and centrifuged at 25 °C, 10,000 x g for 1 min. After centrifugation, the column was placed in a clean collection tube. After adding 500µl Wash Buffer 2 and centrifuging at 21,000 g for 3 min at 25 °C, the collection tube was discarded and the column was placed in a clean collection tube. Then, 50µl elution buffer was added and incubated at 25 °C for 1 min. After incubation, it was centrifuged at 21,000 g at 25 °C for 1 min, and the spin column was discarded.

Hotspot Panel Sequencing: The genes included in the panel we used in our study are common TSGs and oncogenes (Table 1).⁹

Table 1. Tumor suppressor genes and proto-oncogenes used in our study.

PROTO-ONCOGENES	TUMOR SUPPRESSOR GENES
ABL1, AKT1, ALK, BRAF, CTNNB1, CSF1R, EGFR, ERBB2, ERBB4, EZH2*, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HRAS, IDH1, JAK2, JAK3, IDH2, KDR, KIT, KRAS, MET, MPL, NOTCH1*, NRAS, PDGFRA, PIK3CA, PTPN11, RET, SMO, SRC.	APC, ATM, CDH1, CDKN2A, EZH2*, FBXW7, HNF1A, MLH1, NOTCH1*, NPM1, PTEN, RB1, SMAD4, SMARCB1, STK11, TP53, VHL.

*EZH2 and NOTCH1 are genes that can exhibit both oncogenic and tumor suppressor functions in melanoma, depending on the binding and mutation type. The classification in this table is based on their predominant biological effects in advanced/metastatic melanoma.

The Ion AmpliSeq™ Cancer Hotspot Panel (Thermo Scientific, 4475346) is a primer pool for the preparation of amplicon libraries from ‘hotspot’ regions that are frequently mutated in human cancer genes.¹⁰ Selected hotspot SNVs (>2800 mutations in the COSMIC database) in specific genes were simultaneously multiplex-sequenced in both forward and reverse directions using the Ampliseq Cancer Hotspot DNA Panel v2. DNAs were diluted to 5 ng/μl. After spectrophotometric reading, fluorometric measurement was performed with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851). Ion Library Kit Plus (ThermoFisher Scientific, 4488990) was used for amplification of more than 50 target gene regions in 2 different primer pools. Multiplex amplification was performed by combining sample DNAs previously distributed into the separated wells on the PCR plate. DNA fragments were modified to form sticky ends with the FuPa enzyme for the addition of index and adapter sequences. DNA fragments were amplified simultaneously using the DNA ligase enzyme. In order to read, they were barcoded with adapters and index sequences, and the adapters and barcodes were attached to the amplicons. The library was purified using magnetic beads, and emulsion PCR, enrichment and chip loading processes were performed automatically using the Ion Chef™ Instrument (ThermoFisher Scientific, 4484177). Barcoding and adaptering processes and reading of DNA sequences of approximately 200-225 bp in length were completed by using the Ion S5 520 chip in the Ion Torrent™ Ion S5 device. By the chip with a total reading capacity of 5 million, 4 DNA samples were sequenced simultaneously with an average reading depth of 1000X.

Data Analysis: Approximately 225bp single-end sequence raw data (fastq or UBAM) obtained from the next-generation sequencing platform were used. The 5’ and 3’ ends of the DNA sequence data were trimmed to certain lengths (primer, A adapter, P1 adapter and Barcode adapter) using the quality parameters previously determined in the system, and the reading results of the sequence were aligned to the reference human genome (hg19/GRCh37.p13) sequence using Torrent Suite V5.8.4 software. Variant identification was performed with Torrent Variant Caller. In the filtering created on the Ion Reporter™ platform, unreliable variations (<20%) were eliminated according to the percentage of benign variation detected, and unreliable variations (<100x) were eliminated according to the reading depth of the detected variations and analyzed. Afterwards, whether the detected variations had been previously identified was investigated using in silico programs and house databases such as “dbSNP”, “HGMD”, “5000 Exomes” and “1000 genomes”, and “COSMIC”. More detailed classification of the variants was provided by referencing the records in COSMIC (Catalogue of Somatic Mutations in Cancer) and dbSNP databases. In addition, in silico analyses (e.g. SIFT, PolyPhen-2) were used to evaluate the possible pathogenicity of the variants. The variants were interpreted according to the ACMG/AMP 2015 guidelines.¹¹

RESULTS

Mutated genes and detected mutation types in WM115 primary melanoma cell line; a total of 9 mutations were detected in 6 genes: 3 in the ERBB4 gene, 2 in the BRAF gene and 1 each in the FGFR3 gene, FLT3 gene, and TP53 gene. The cell pathways associated with the genes with mutations are as indicated in Table 2: ERBB signaling pathway, MAPK signaling pathway, cell proliferation, neoangiogenesis, regulation of actin cytoskeleton, RET signaling pathway, cell cycle, apoptosis, DNA repair mechanism and androgen receptor signaling pathway.

Table 2. Hotspot mutations detected in WM115 primary melanoma cell line.

Genes	Transcript	Mutation	Amino acid conversion	Mutation classification	Associated pathway ¹²
ERBB4	NM_005235.3	c.884-8_884-7delTT	-	VUS	ErbB signaling pathway
		c.884-21_884-18delinsTTTC	-	VUS	
		c.421+58A>G	-	Benign	
BRAF	NM_004333.6	c.1799_1800delTGinsAT	p.Val600Asp	Patojenik	MAPK signaling pathway
FGFR3	NM_000142.4	c.1799T>A	p.Val600Glu	VUS	Cell proliferation, neoangiogenesis, Regulation of the actin cytoskeleton
		c.1205C>T	p.Pro402Leu		
MET	NM_001127500.3	c.3029C>T	p.Thr1010Ile	VUS	RET signaling pathway, Focal adhesion
FLT3	NM_004119.3	c.1310-3T>C	-	Benign	Cell proliferation, Antiapoptotic effect
TP53	NM_000546.5	c.215C>G	p.Pro72Arg	Benign	Cell cycle, Apoptosis, DNA repair mechanism, AR signaling pathway

As seen in Table 3, mutated genes and detected mutations types in SK-MEL-30 metastatic melanoma cell line; a total of 26 mutations were detected in 10 genes: 1 in the VHL gene, 1 in the FBXW7 gene, 7 in the MET gene, 2 in the SMO gene, 2 in the ATM gene, 2 in the SMAD4 gene, 3 in the PIK3CA gene, 4 in the NOTCH1 gene, 2 in the KRAS gene, 1 in the ERBB2 gene, and 1 in the SMARCB1 gene. The cellular pathways associated with the mutated genes are ERK, ERBB, Notch and Hedgehog signalling pathways, Homology Directed Repair (HDR) pathway, canonical and non-canonical TGF-Beta signalling pathway, RET signalling pathway, Sonic Hedgehog signalling pathway. Our results reflect only a part of the complete genetic profile, especially considering the heterogeneity in metastatic tumors. Advanced genomic analyses might provide a better understanding of genetic heterogeneity and clonal evolution.

Table 3. Hotspot mutations detected in SK-MEL-30 metastatic melanoma cell line.

Genes	Transcript	Mutation	Amino acid conversion	Mutation classification	Associated pathway ¹²
VHL	NM_000551.4	c.259_261delGTAAinsTTG	p.Val87Leu	Possible Pathogenic	HIF
FBXW7	NM_033632.3	c.1469_1470delCAinsGC	p.Thr490Ser	VUS	Notch signaling pathway
MET	NM_001127500.3	c.507_508delCAinsAG	p.[Pro169=;Ser170Gly]	VUS	RET signaling pathway, Focal adhesion
		c.483A>G	p.Ile161Met	VUS	
		c.563T>A	p.Val188Glu	VUS	
		c.1028T>A	p.Phe343Tyr	VUS	
		c.1048A>C	p.Lys350Gln	VUS	
		c.1116C>G	p.Asp372Glu	VUS	
		c.2942-20T>C	p.?	VUS	
SMO	NM_005631.5	c.584_585delTGinsCA	p.Val195Ala	VUS	Hedgehog signaling pathway
		c.575A>G	p.Gln192Arg	VUS	
ATM	NM_000051.3	c.5779_5780delATinsGC	p.Ile1927Ala	VUS	HDR signaling pathway
		c.5785A>G	p.Asn1929Asp	VUS	
SMAD4	NM_005359.6	c.516_517delGTinsAC	p.[Leu172=;Ser173Pro]	VUS	TGF-Beta signaling pathway
		c.589A>G	p.Thr197Ala	VUS	

Continuation of Table 3

Genes	Transcript	Mutation	Amino acid conversion	Mutation classification	Associated pathway ¹²
PIK3CA	NM_006218.4	c.1251+10T>C c.1251+18G>T c.1251+30T>C	p.? p.? p.?	VUS VUS VUS	RET signaling pathway
NOTCH1	NM_017617.5	c.4793G>A c.4763G>A c.4740G>C c.4738A>C	p.Arg1598His p.Ser1588Asn p.Met1580Ile p.Met1580Leu	VUS VUS VUS VUS	Notch signaling pathway
KRAS	NM_033360.4	c.112-6T>C c.112-10C>A	p.? p.?	VUS VUS	RET signaling pathway
ERBB2	NM_004448.3	c.2307+16C>T	p.?	VUS	ErbB signaling pathway
SMARCB1	NM_003073.5	c.1119-41G>A	p.?	Benign	Cell cycle, WNT, Hedgehog signaling pathway

DISCUSSION AND CONCLUSION

Melanoma is the most lethal type of skin cancer. It is a heterogeneous disease resulting from a combination of genetic risk factors and accumulated environmental factors.^{13,14} In this study, we aimed to perform a comparative analysis of hotspot mutation profiles in primary (WM115) and metastatic (SK-MEL-30) melanoma cell lines. In our study, mutations in ERBB4, BRAF, FGFR3, MET, FLT3 proto-oncogenes, and TP53 tumor suppressor genes were detected in WM115 the primary melanoma cell line. Variants of uncertain significance (VUSs) were identified in ERBB4, FGFR3, and MET, benign mutations in FLT3 and TP53, and a pathogenic mutation in a single BRAF gene.

In a study, it was found that melanomas found in areas with minimal sunlight exposure usually showed BRAF or NRAS mutations. In contrast, melanomas found in regions with chronic sunlight exposure were mostly associated with TP53 mutations. These results suggest that melanoma-associated mutations can be triggered over time by somatic or environmental factors.¹⁴ Bastian et al. suggested that melanoma usually involves a gain-of-function mutation in the NRAS, HRAS, BRAF, KIT, GNAQ, GNA11, ALK, ROS1, RET, and NTRK1 genes and loss of TSGs such as CDKN2A, TP53, PTEN or BAP1 in the second and third stages.¹⁵ Mutations in the BRAF proto-oncogene are implicated in various cancers and play a key role in the MAPK/ERK signaling pathway, which regulates cell growth, migration, and proliferation. BRAF mutations are classified into three types: Class I (V600E), acting as constitutively active monomers; Class II, forming active dimers; and Class III, with decreased kinase activity. Class I mutations are the most common in melanoma.^{16,17} In melanomas, the somatic BRAF mutation rate is the highest among cancer types, and pathogenic variants in the BRAF gene are observed in approximately 50% of patients affected by melanoma. BRAF mutations are more common in young patients with short-term sun exposure than in chronically exposed individuals. V600E accounts for 70-88% of BRAF-positive melanomas. The second most common variant is p.V600K, which replaces valine with lysine and occurs at a frequency of approximately 10-20%.¹⁸ In our study, we detected two pathogenic class I BRAF mutations in the WM115 primary melanoma cell line, which are the conversion of valine to glutamic acid and valine to aspartic acid. This result is consistent with previous reports showing that BRAF mutations are among the most common driver alterations in melanoma. The detection of BRAF V600 mutations supports the technical validity of the mutation profiling approach used in this study.

Our study observed a broader mutation profile in the SK-MEL-30 metastatic melanoma cell line compared to the WM-115 cell line. Most of the identified variants were classified as VUS, affecting multiple genes, including MET, SMO, PIK3CA, NOTCH1, KRAS, ERBB2, FBXW7, ATM, and SMAD4. A possible pathogenic mutation was found only in VHL.

The VHL tumor suppressor gene encodes pVHL, which regulates hypoxia-inducible factors (HIFs) and mediates cellular adaptation to oxygen deficiency.^{19,20} pVHL is part of a complex involved in the ubiquitination and degradation of transcription factors that play various roles in cells, which forms the basis of cellular adaptation to oxygen deficiency.²¹ In hypoxic conditions or the absence of functional pVHL, HIF α accumulates and forms heterodimers with HIF1 β . These heterodimers translocate to the nucleus, where they bind to hypoxia-responsive

elements (HREs) and induce transcription of genes involved in adaptation to hypoxia.^{20,22} Expression of HIF-1 α is high in melanoma, plays an important role in driving malignant transformation in cancer cells, and is considered to be an important biomarker for liver metastasis.²³ In our study, 1 possible pathogenic mutation caused by the conversion of valine to leucine was detected in the SK-MEL-30 metastatic melanoma cell line. The presence of a potentially pathogenic VHL variant suggests a potential association with hypoxia-related pathways, given the known role of VHL in regulating hypoxia-sensitive factors. However, a single variant is insufficient to confirm HIF activation or its role in metastasis, and further HIF-focused validation is needed.

In the WM115 primary melanoma cell line, 4 VUS were detected. In the SK-MEL-30 metastatic cell line, 24 VUS were observed. It remains unclear whether VUSs contribute to the disease or whether they are rare variants with little or no association with cancer risk. Comprehensive sequencing approaches have increased the number of identified VUSs. However, limited functional and clinical data on these variants pose significant challenges for genetic counsellors and clinicians in patient management.²⁴ In both cell lines, VUS were distributed across multiple signaling pathways, including NOTCH, RET, and Hedgehog. In metastatic melanoma, most VUS findings are detected in the RET and NOTCH pathways. While these pathways are implicated in cancer progression, our data do not allow determination of whether these variants alter pathway activity or function. These results provide a descriptive framework for the distribution of genetic alterations in the metastatic cell line, independent of the functional effects of the variants. Therefore, these findings should be interpreted as descriptive of variant distribution rather than indicative of pathway dysregulation.

This study has several limitations. Only two melanoma cell lines were used in the analysis. Most of the identified variants were classified as VUS, which limited the functional interpretation. Furthermore, no experimental validation was performed to evaluate the biological effect of the identified variants on pathway activity. Therefore, the findings of this study should be considered descriptive and hypothetical.

In conclusion, this study presents a comparative overview of hotspot mutation profiles in primary and metastatic melanoma cell lines. The detection of BRAF class 1 mutations in the primary cell line is consistent with known melanoma genetics; however, the higher prevalence of VUS in the metastatic cell line compared to the primary cell line indicates increased mutational heterogeneity. The distribution of variants in multiple signaling pathways highlights mutational differences between disease stages. Most melanoma genomic studies focus on clinically targetable genes. In contrast, this study compares primary and metastatic cell lines through a holistic approach, including both pathogenic variants and VUS. The higher VUS burden and pathway-level distribution in the metastatic line underscore increasing genetic heterogeneity during melanoma progression. Overall, these findings emphasize differences in mutation profiles between primary and metastatic melanoma cell lines and provide a basis for future studies incorporating functional validation and larger sample sizes to better understand the biological relevance of these variants.

Ethics Committee Approval: Ethical approval for the study is not required. In this study, secondary cell culture was used.

Conflict of Interest: No conflict of interest was declared by the authors.

Authors' contributions: Concept – ES, CD; Supervision – ES, CD, OOG; Materials – CD, ES; Data Collection and/or Processing – ES, CD; Analysis and/or Interpretation – ES, CD, OOG; Writing –DA, SS.

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