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Research Article / Araştırma Makalesi

The importance of *FISH* Test Targeting *EGFR*, *CCND1* and *RREB1* Genes in Differentiating Malignant Melanomas from Melanocytic Nevus

Malign Melanomlar ile Melanositik Nevüs Ayrımında EGFR, CCND1 ve RREB1 Genlerini Hedef Alan FISH Testinin Yeri.

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Abstract

Melanocytic nevus (MN) may on occasion be difficult to distinguish from malignant melanoma (MM) histopathologically. Fluo-rescent in situ hybridization (FISH) has been demonstrated to be of use for the diagnosis of melanocytic neoplasms of the skin. In this study, the effectiveness of the standart melanoma *FISH* test (4-way probe targeting *RREB1, CCND1, MYB* genes and centro-mere 6) and additionally probes, targetting *EGFR*, *TP53*, *MDM2* and *P16* genes, in differentiating melanomas from melanocytic nevi were investigated. Standard *FISH* test was performed on 24 MM and 24 MN samples, but *EGFR*, *TP53*, *MDM2* and *TP53* gene copy numbers were investigated in 16 of 24 MM and 24 MN using FISH method. The incidence of FISH-detected positive genomic copy aberrations (4-way probe, and others) was determined as 83,3% in 24 MM cases, and 5,2% in 24 MN. Statistically significant differences were found between the MM and MN groups in terms of *CCND1*, *RREB1*, *EGFR* amplifications ($p<0.001$, $p<0.05$, $p<0.05$), but there was no association between histopathological features and detected abnormalities ($p>0,05$). In additio-nally, all 5 acral lentiginous melanomas, could be analysed, had *EGFR* amplifications. In conclusion, *CCND1*, *RREB1*, and *EGFR* amplifications have diagnostic significance for MM. The FISH test is very effective in terms of its use as an adjunct to histopatho-logical methods. But centromere controlled probes should be used to avoid false positive results.

Keywords: FISH, *EGFR, CCND1, RREB1,* Malignant melanoma, Melanocytic nevus

Özet

Özet: Melanositik nevüs (MN) örneklerini histopatolojik olarak malign melanomdan (MM) ayırmak bazen zor olabillmektedir. Floresan in situ hibridizasyon (FISH)'in cildteki melanositik neoplazmların teşhisinde kullanıldığı kanıtlanmıştır. Bu çalışmada standart melanom FISH testinin (*RREB1, CCND1*, *MYB* genlerini ve sentromer 6'yı hedefleyen 4 yollu prob) ve ayrıca *EGFR*, *TP53, MDM2* ve *TP53* genlerini hedefleyen probların melanomları melanositik nevüslerden ayırmadaki etkinliği araştırılmıştır. 24 MM ve 24 MN örneklerine standart FISH testi uygulandı. Ancak *EGFR, TP53, MDM2* ve *P16* gen kopya sayıları FISH yöntemi kullanılarak 24 MN ve 16/24 MM örneklerinde incelendi. Floresan in situ hibridizasyon ile saptanan pozitif genomik kopya aberasyonlarının (4 yollu prob ve diğerleri) görülme sıklığı 24 MM vakasında %83,3 ve 24 MN'de %5,2 olarak belirlendi. Malign melanom ve MN grupları arasında *CCND1*, *RREB1*, *EGFR* amplifikasyonları açısından istatistiksel olarak anlamlı farklılıklar bu-lundu ($p<0,001$, $p<0,05$, $p<0,05$), ancak histopatolojik özellikler ile saptanan anormallikler arasında ilişki saptanmadı ($p>0,05$). Ek olarak, 5 akral lentijinöz melanomun tümü analiz edilebildi ve hepsi *EGFR* amplifikasyonu açısından pozitifti. Sonuç olarak, *CCND1*, *RREB1* ve *EGFR* amplifikasyonları MM için tanısal öneme sahip olduğu ve FISH testinin, histopatolojik yöntemlere ek olarak kullanılmasının etkili olabileceği sonucuna varılmıştır. Ancak, yanlış pozitif sonuçlardan kaçınmak için sentromer kontrol-lü problar kullanılması gerekliliği gözlenmiştir.

Anahtar Kelimeler: FISH, *EGFR, CCND1, RREB1*, malign melanom, melanositik nevüs

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1. Introduction

Melanoma is a malignant transformation of melanocytes with extremely poor prognosis. It is a disease with increasing incidence and is the most fatal form of skin cancer. According to the World Health Organization statistics, it is also estimated that the number of newly diagnosed melanoma cases and the deaths from melanoma will be about 500,000 and 106,000, respectively until 2040. Genetic, phenotypic and environmental risk factors all contribute to the susceptibility to melanoma. (1, 2).

Histopathological examination is the gold standard to discriminate a malignant melanoma (MM) from a melanocytic nevi (MNs). However, some difficulties arise in differentiating MNs from MMs because of the atypical cytological and architectural structures. Some of these lesions are considered as borderline melanocytic tumors. Since clinicians often expect a definitive diagnosis as is either benign or malignant lesion, some uncertain reports even from experienced pathologists cause significant frustrations which in turn has direct implications for patient management (3-5).

It is well-known that both the *BRAF* and *NRAS* oncogenic mutations are common molecular markers involved in the development and progressions of MMs and MNs. However, there is a requirement for helpful diagnostic markers to accurately classify melanocytic tumors. The major difference between MN and MM is the presence of numerous and recurrent chromosomal imbalances in MMs. The copy number gains of *HRAS* (Harvey rat sarcoma viral oncogene homolog) locus, located on chromosome 11p15, is an exception seen in the Spitz nevi (SN), which has not been reported to date in melanomas (6).

In recent years, Fluorescent *In Situ* Hybridization (FISH) analysis has been used in the diagnosis of specimens which cannot be clearly determined according to histopathological criteria, between conventional melanoma or to differentiate Spitz type. Numerous changes in the number of chromosome copies may indicate flaws in the TP53 pathway. Copy number changes of *MDM2* and *CDKN2A* in *TP53* path may affect TP53 function (7). Additionally, *EGFR* is an important therapeutic target in cancer. Therefore, in our study, we aimed to investigate the effectiveness of both the classic melanoma FISH test and four additional probes targeting *CDKN2A*/cen9, *EGFR*/Cen7, *TP53*/Cen17, and *MDM2*/Cen12 genes in distinguishing MM samples from MNs.

2. Materials and Methods

2.1. Case Group

Our study group consisted of 48 archive samples of 33 women and 15 men cases diagnosed between 2008-2013. The age of the patients ranged from 20 to 88 years (mean 51,75±16 years). This study was conducted according to the guidelines that were declareted in the Declaration of Helsinki, and approved by the Clinical Practice Ethics Committee (2011-17). Each individual provided signed consent form.

The histopathological features were individually assessed by a dermatopathologist. Of total 48 samples, histopathological examinations grouped 24 samples as MM (8 nodular, 3 superficial spreading, 6 lentigo maligna, 6 acral lentiginous melanoma and 1 nevoid type MM) whereas the remaining 24 were classified as MN (12 dysplastic compound nevi, 5 compound nevi, 2 SN, 3 intradermal nevi, 1 junctional nevi compatible with SN and a case of a non-subtype nevi) groups.

2.2. Fluorescent *In Situ* hybridization

Fluorescent *in situ* Hybridization analysis was performed using a four-probe assay from Abbott Molecular, Inc. (01N89-020) targeting Ras responsive element binding protein-1 (*RREB1*) on 6p25 (Vysis ®LSI ® *RREB1*-Spectrum Red), V-*MYB* myeloblastosis viral oncogene homolog (*MYB*) on 6q23 (Vysis®LSI® *MYB* - Spectrum Gold), *CCND1* on 11q13.3 (Vysis®LSI® *CCND1*-Spectrum Green) and centromeric enumeration probe control for chromosome 6 (Vysis®LSI® CEN6-Spectrum Aqua). Additional probes

including cyclin dependent kinase inhibitor 2A (*CDKN2A*) on 9p21 and centromeric control for chromosome 9 (Cytocell® FISH probes *CDKN2A/Cen9),* Epidermal Growth Factor Receptor (*EGFR*) on 7p11.2-p12 *(*Vysis®LSI® *EGFR* SpectrumOrange/Vysis CEP 7 SpectrumGreen), *TP53* tumor suppressor gene on 17p13 (Zyto*Light* ® SPEC *TP53*/CEN 17 Dual Color) and *Mouse double minute 2* homolog (*MDM2*) also known as E3 ubiquitin-protein ligase *MDM2* on 12q15 *(*Zyto*Light* ® SPEC *MDM2*/CEN 12 Dual Color) were applied on the samples.

Following deparaffinization and rehydratation of 4µm thick sections prepared from formalin fixed and paraffin embedded tissues, FISH was performed with a hybridization automation (ThermoBrite; Abbott Molecular). After co-denaturation at 75°C during 10 min, the probes and the target DNA were allowed to hybridize at 37°C overnight. Then, the excess probes and non-specific hybridizations were eliminated by stringent washings in a bath with 2X saline sodium citrate and Tween 20 at 72°C. Before microscopic evaluation nuclei were counterstained with DAPI (40,6-diamidino2-phenylindole) in antifade solution (Vector Laboratoratories, USA).

2.3. FISH analysis

The areas to be analyzed in the samples of all cases were determined by dermatopathologists. At least 30 nuclei in each area were analyzed. A case was considered positive for melanoma if any of the following criteria was met (8): 1) Gain in 6p25 (*RREB1)* in >29% (>2 spots/nucleus) nuclei, 2) gain in 6p25 (*RREB1*) relative to CEN6 in 55% nuclei, 3) relative loss of 6q23 (*MYB*) compared to CEN6 in >40% nuclei, 4) gain in 11q13.3 (*CCND1*)(>2 signals/nucleus) in >38% nuclei and 5) >33% nuclei with a homozygous loss of *CDKN2A* on 9p21 (9) 6) >15% gain in *EGFR* on 7p11(10). The cut off values for *TP53* deletion and *MDM2* amplification were accepted as 8% according to conditions of our laboratory and manufacturer's recommendation.

2.4. Statistical analysis

In order to compare the differences observed in terms of 4-way probe, and *EGFR, P16, TP53, MDM2* aberrations ratio between malignant and benign tumour tissues, the X^2 statistical test was used in IBM SPSS 21.0 statistical program, and p values were calculated according to the Fisher's Exact test. The relationship between the histopathological features (breslow thickness, clark level, ulceration, mitosis rate, lymphocyte infiltration, growth phases) of the patients and the aberrations observed were evaluated by the $\chi 2$, T-Test and Mann Whitney U test, using the IBM SPSS 21.0 package program. $p < 0.05$ were considered statistically significant. The posterior power analysis were performed for statistically significant results.

3. Results

Genes regions (*EGFR*, *MDM2*, *TP53* and *P16*) were able to examined in 16 of 24 MM lesions, because of insufficient tissue sections. The incidence of FISH-detected positive genomic copy aberrations (4-way probe, and others) was determined as 83.3% (20/24) in 24 MM cases.

Among 20 MM lesions, positive FISH results, one FISH criterion was seen in 9, two criteria in 6, three criteria in 2 and four criteria including *CCND1* amplification, *MYB* deletion, *RREB1* amplification and gain in *RREB1* relative to CEN6 in three lesions. As seen in Table 1, amplification in *RREB1* was found in 10 lesions, gain in *RREB1* relative to CEN6 in 6 lesions, loss of *MYB* in 9 lesions, gain in *CCND1* nuclei in 13 lesions, and homozygous loss of *CDKN2A* in 1 lesion. Moreover, relative to each of their corresponding centromere signals, gain in *EGFR* and *MDM2*, and loss of *TP53* gene, were observed in 7, 3 and 1 lesions, respectively. Images of positive FISH results are shown in figure 1.

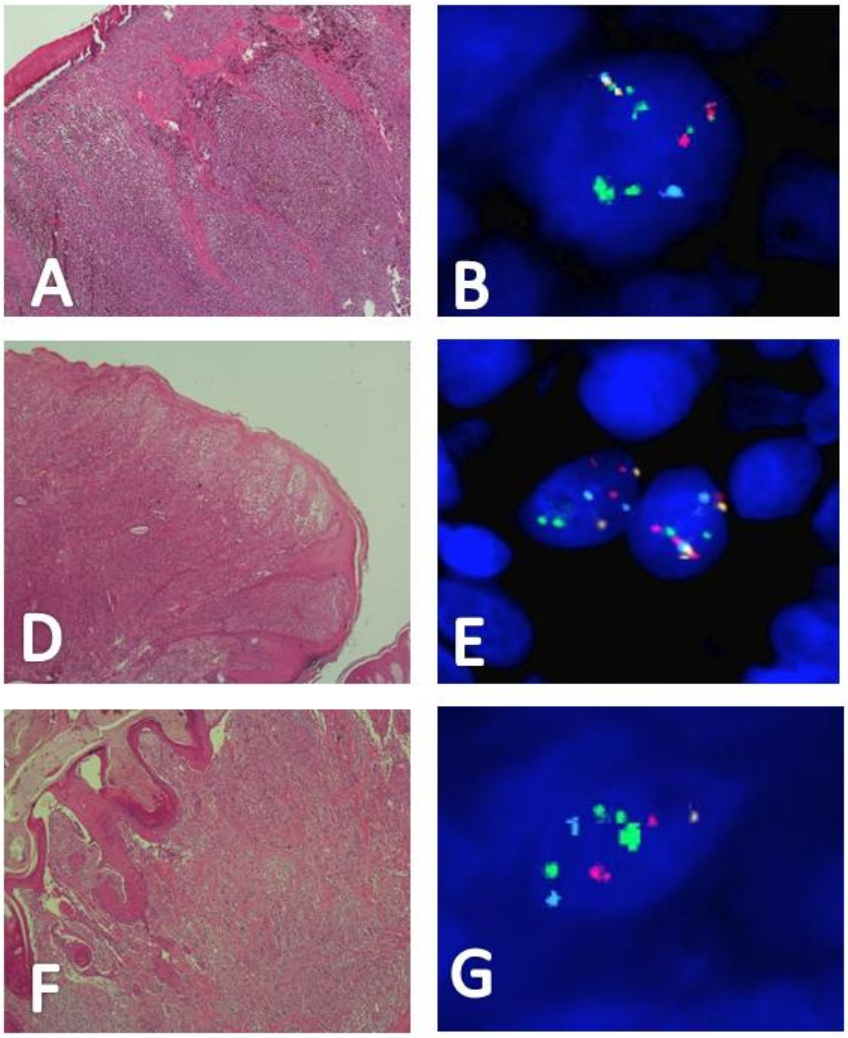

Figure 1. *Views of X40 hematoxylin and eosin (H&E) stained sections and X100 interphase fluorescence in situ hybridization (FISH) (green= 11q13, red=6p25, aqua=6q23, gold= cen6). A, B) H&E and FISH images of sample 2. FISH image illustrates a CCND1 amplification profile C,D) H&E and RREB1 amplification FISH images of sample 1. E,F) Images of H&E and FISH relative to sample 7. FISH analysis on the recurrence shows loss of MYB, and gain of CCND1 and RREB1.*

According to the results of FISH study in MN cases, genomic copy aberrations was determined in 7 MN cases (Table 1). Gain in *MDM2* was found in 2 lesions. Amplification in *RREB1*, deletion in *MYC*, and deletion in *CDKN2A* were detected in one case each. The copy number gain in 6p25 and 6p25 relative to CEN6 as well as deletion in locus 6q23 were positive in one of the SN (case #32). Also, both *MYB* deletion and *MDM2* gene amplifications were detected in the case #41.

When MM and MN groups were compared respect to FISH-detected signal abnormalities, statistically significant differences were found in terms of *CCND1*, *RREB1* and *EGFR* genes amplifications between MM and MN cases: $p<0.001$, $p<0.05$, $p<0.05$, respectively. The posterior power of the relation between group and *CCND1, RREB1* and *EGFR* amplifications were respectively 98.8%, 76%, 97.4%. However, no correlation was found between histopathological features and abnormalities detected by FISH (Table 2).

All acral lentiginous melanomas, 6 of 8 nodular and 5 of 6 lentigo malignant melanomas lesions had at least one positive

FISH criterion. Additional FISH-detected loci were analysed in five of 6 acral lentiginous melanomas and all analysed lesions had *EGFR* gene amplifications. The loss of *TP53* was only seen in acral lentiginous melanoma lesion.

In addition, there was no statistically significant difference between MM and MN cases in terms of gender and age.

4. Discussion

Histopathology is the gold standard for diagnosing melanocytic lesions, but distinguishing benign versus malignant is not always clear histologically. Therefore, studies are ongoing to determine the molecular markers for the differentiation of benign and malignant lesions. Although the effectiveness of 4-way FISH in distinguishing benign from malignant pigmented lesions is well documented in the literature, the validity of FISH technique in our laboratory was evaluated in the differentiation of benign and malignant lesions by including additional gene regions (11, 12).

In previous studies (8, 13-16), anomaly detection rates by FISH with a 4-way probe have been reported to be between 78-100%. In our study, the specificity and sensitivity of the FISH technique with 4-way probe in differentiating benign and malignant lesions was found to be 83.33% interestingly, and it was observed to be compatible with the literature (Table 3). In MM and MN cases evaluated with 4-way probe, statistically significant differences were found especially in terms of *RREB1* and *CCND1* gene amplifications. No abnormalities were observed in four MMs. Gerami et al. stated that positive FISH data in lesions is the determinant for the definitive diagnosis of melanoma, whereas the negative FISH test has limited diagnostic value (17). In another study, it was stated that obtaining a negative result with the 4-way FISH set did not exclude the diagnosis of MM and that there could be anomalies in different regions from the tested loci (5). This is one of already known disadvantages of FISH technique.

Although the most common anomaly in the literature was reported as *RREB1* amplification, the predominantly seen abnormality in our MM series was *CCND1* gene amplification (54.2%) (Table 4). In the literature, *CCND1* amplification detection rates have been reported between 0-66%. However, our knowledge about the reason for this diversity is limited. Previous studies have shown that *CCND1* amplification has been detected in chronic sun-exposed MM cases (5, 18, 19). Chronic exposure to sun could not be evaluated in our cases. It is well known that the protein encoded by this gene belongs to the highly conserved cyclin family and forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. The amplification and overexpression of this gene alters cell cycle progression and observed frequently in a variety of tumors. It is previously demonstrated that *CCND1* amplification is a frequent event in the acral lentiginous MMs (7). In consistent with this, *CCND1* amplification in our study was also higher in acral lentiginous MM (5/6) compared to other types.

On the other hand, incidance of *TP53* mutations in melanomas is %16.(20) In our case series, *TP53* deletion was observed in only one lesion and it was acral lentiginous melanoma. *CCND1* and *EGFR* amplifications and *TP53* deletion were detected in this lesion. High levels of structural rearrangements and copy number changes in acral melanoma may be associated with impaired guard function of the *TP53* pathway (7). However, *TP53* deletion was not detected in other lesions with multiple anomalies. The possibility of point mutation could not be ruled out because it was analyzed by FISH, which is a limitation of our study.

In our study, when the previously reported cut-off values were used, a significant difference was found between MN and MM cases in terms of *RREB1* amplification. Of 10 (41.6%) MM lesions with *RREB1* amplification, 6 (25%) had also *RREB1*/Cen6 amplification. Although our *RREB1* amplification detection rate was consistent with the literature, the *RREB1*/Cen6

amplification ratio was lower. However, poliploidy is frequently observed in MM and SN cases and these polyploidies are considered as non-diagnostic. In addition, tetraploid cells may cause false positive results in the FISH assay, which is a major cause of the rate differences between studies.

In melanoma, data on *EGFR* protein expression are conflicting. Previously, it has been stated that *EGFR* gene amplification to be linked with metastasis, thicker tumor, and ulceration and therefore worse prognosis (10, 21, 22). In the presented study, we detected *EGFR* amplification in 7 of 16 MM cases (43.8%), and this alteration was predominantly seen in acral lentiginous melanoma. The acral lentiginous melanoma is associated with a worse prognosis than cutaneous MM overall. Previously reported that *EGFR* polysomy was associated with thicker tumors and bad survival. *EGFR* appears to be involved in progression and metastasis of a subset of melanomas (21). Targeting *EGFR* could therefore represent a therapeutic option for these melanomas. Recently, Koroknai et al. also stated the hypermethylation in the gene body of the *EGFR* gene is positively correlated with its overexpression and might be one of the key changes during the development of malignant melanoma cells (23).

In addition, we found that the *CCND1* amplification was also positive in 6 of 7 lesions with *EGFR* amplification positive. In the study of Katunaric et al., they investigated the diagnostic and prognostic significances of nuclear *EGFR* expression and its correlation with *CCND1* expression in nodular melanomas. They mentioned that *EGFR* and *CCND1* expressions were of no diagnostic importance and there was no correlation between them (24). *CCND1* is located in the downstream of the *EGFR* pathway. The team argued the reason of not having been able to find a correlation was due to the fact that *CCND1* was induced by a different mechanism. However, as mentioned previously, the prevalence of *CCND1* anomaly was also high in our acral lentiginous melanomas. Therefore, our data supports the role of these genes in the progression of the molecular background of these type melanomas associated with poor prognosis.

Previously, melanomas from sun-protected tissues (mucosal, acral melanomas) were thought to have similar molecular characteristics. However, Zhou et al. showed that repetitive clustered rearrangements in mucosal melanomas were mostly on chromosomes 11 and 17, whereas chromosome 12q specific amplifications were predominantly found in in acral lesions. CDK4, *MDM2* and AGAP2 loci are localized at 12q13–15. Zhou et al. reported that *MDM2* gene amplification was positive in 50% of melanomas. The prevalence in our cohort was lower. *MDM2* gene amplification was positive in 3 of the 16 analyzed melanomas, and all positive ones were in the acral lentiginous melanoma subtypes that is consistent with the recent data (25).

Chromosome 9p21 losses are reported as one of the most common chromosomal abnormalities in the literature. In particular, it plays a role in homozygous deletion processes. However, there are controversial data in the literature regarding *CDKN2A* deletions and the relationship between sporadic melanoma progression (26, 27). *CDKN2A* gene deletions are more common in spitzoid melanoma and familial melanoma (12). In the study, one case was found in each group and it was not interpreted as significant. But abnormal MN cases should be followed up for malignant progression.

On the other hand, It is interesting that *MYB* gene deletions were seen in 3 MN tissues. This is a contradictory finding with the literature because *MYB* deletion has not been previously reported. Gerami et al. examined molecular characteristics of 75 atypical spitz tumor and they reported that the gains in 11q23 and 6p25 loci were associated with poor prognosis, but the clinical findings of patients with 6q23 deletion was suggested to be non-aggressive (28). Tissue sections of 3 MN cases with *MYB* deletion were re-evaluated histopathologically according to World Health Organization Classification of skin tumours published in 2018, but no change was observed in pathological

diagnoses. However, the lesion #32 had not only *MYB* deletion, but also *RREB1* and *RREB1*/Cen6 amplifications were positive and it was stated in its pathological examination report that the risk of MM was high and should be closely monitored. This suggests that the effect of 6q23 losses should be investigated in the progression of melanocytic lesions to malignant.

Melanocytic nevi lesions detected *MDM2* amplification were re-examined histopathologically, and results supported the previous diagnosis. In the literature, it is stated that *MDM2* amplifications are mostly related with tumor progression, but early stage marker in the acral melanomas (29). Therefore, cases whose lesions with positive *MDM2* amplification should be further analyzed.

In conclusion, it has been revealed that *CCND1, RREB1* and *EGFR* amplifications have diagnostic meanings for malignant melanoma cases. *RREB1* signals more than 2 and *RREB1*/Cen6 negativity indicate polysomy and *RREB1* may result in false positive diagnosis without checking centromer signals. For this reason, centromere-controlled probes should be used during the amplification assesment analysis. As a result, FISH test with centromer controlled probes targeting *EGFR*, *CCND1* and *RREB1* genes can be used as an auxiliary test in the diagnosis of melanomas.

Fluorescent *In Situ* Hybridization markers were effective in the definitive diagnosis of MM and MN lesions. We also think that MN cases that are positive for known malignancy-associated markers should be closely monitored clinically. Yet the FISH technique can be used as an adjunct to the gold standard histopathological evaluation. However, due to the advancement of molecular technologies, we believe that the molecular mechanisms based on the understanding of the underlying molecular mechanisms and the identification of new markers will lead to a definitive diagnosis.

Statement of Ethics

This study was conducted according to the guidelines that were declareted in the Declaration of Helsinki, and approved by the Clinical Practice Ethics Committee (2011-17). Each individual provided signed consent form.

- *Study of In Situ Hybridization in Malignant Melanoma. 10th European Cytogenetics Conference (Özet Bildiri/Poster 2015).*
- *Malign Melanom Olgularında Moleküler Sitogenetik Çalışmalar. 2. Ege Hematoloji Onkoloji Kongresi (Özet Bildiri/Poster2016).*

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68:394-424.
2. O'Neill CH, Scoggins CR. Melanoma. Journal of surgical oncology. 2019;120(5):873-81.
3. Ferrara GV, C.D. Fluorescence in situ hybridization for melanoma diagnosis: A review and a reappraisal. *Am J Dermatopathol.* 2016;38:253-69.
4. Karen S. McGinnis SRL, David E. Elder, DuPont Guerry, Lynn Schucter, Michaler Ming, Rosalie Elenitsas. Pathology review of cases presenting to a multidisciplinary pigmented lesion clinic. *Arch Dermatol.* 2002;138:617 - 21.
5. North JP, Vetto JT, Murali R, White KP, White CR, Jr., Bastian BC. Assessment of copy number status of chromosomes 6 and 11 by FISH provides independent prognostic information in primary melanoma. *Am J Surg Pathol*. 2011;35:1146-50.
6. Bastian BC, Wesselmann U, Pinkel D, Leboit PE. Molecular cytogenetic analysis of Spitz nevi shows clear differences to melanoma. *J Invest Dermatol.* 1999;113:1065-9.
7. Yeh I, Jorgenson E, Shen L, Xu M, North JP, Shain AH, et al. Targeted Genomic Profiling of Acral Melanoma. *Journal of the National Cancer Institute*. 2019;111:1068-77.
8. Gerami P, Wass A, Mafee M, Fang Y, Pulitzer MP, Busam KJ. Fluorescence in situ hybridization for distinguishing nevoid melanomas from

mitotically active nevi. *Am J Surg Pathol*. 2009;33:1783-8.
9. Rand AJ, Flejter WL, Dowling CA, Brooke LM, Boland GM, Kroshinsky D, et al. Atypical ALK-positive Spitz tumors with 9p21 homozygous deletion: Report of two cases and review of the literature. *J Cutan Pathol*. 2018;45:136-40.
10. Rakosy Z, Vizkeleti L, Ecsedi S, Voko Z, Begany A, Barok M, et al. EGFR gene copy number alterations in primary cutaneous malignant melanomas are associated with poor prognosis. *Int J Cancer.* 2007;121:1729-37.
11. Damsky WE, Bosenberg M. Melanocytic nevi and melanoma: unraveling a complex relationship. *Oncogene.* 2017;36:5771-92.
12. Ferrara G, De Vanna AC. Fluorescence In Situ Hybridization for Melanoma Diagnosis: A Review and a Reappraisal. *Am J Dermatopathol*. 2016;38:253-69.
13. Busam KJ, Fang Y, Jhanwar SC, Pulitzer MP, Marr B, Abramson DH. Distinction of conjunctival melanocytic nevi from melanomas by fluorescence in situ hybridization. *J Cutan Pathol.* 2010;37:196-203.
14. Newman MD, Lertsburapa T, Mirzabeigi M, Mafee M, Guitart J, Gerami P. Fluorescence in situ hybridization as a tool for microstaging in malignant melanoma. *Mod Pathol.* 2009;22:989-95.
15. Newman MD, Mirzabeigi M, Gerami P. Chromosomal copy number changes supporting the classification of lentiginous junctional melanoma of the elderly as a subtype of melanoma. *Mod Pathol.* 2009;22:1258-62.
16. Pouryazdanparast P, Haghighat Z, Beilfuss BA, Guitart J, Gerami P. Melanocytic nevi with an atypical epithelioid cell component: clinical, histopathologic, and fluorescence in situ hybridization findings. *Am J Surg Pathol*. 2011;35:1405-12.
17. Gerami P, Beilfuss B, Haghighat Z, Fang Y, Jhanwar S, Busam KJ. Fluorescence in situ hybridization as an ancillary method for the distinction of desmoplastic melanomas from sclerosing melanocytic nevi. *J Cutan Pathol.* 2011;38:329-34.
18. Colombino M, Sini M, Lissia A, De Giorgi V, Stanganelli I, Ayala F, et al. Discrepant alterations in main candidate genes among multiple primary melanomas. *J Transl Med*. 2014;12:117.
19. Pouryazdanparast P NM, Mafee M, Haghighat Z, Guitart J, Gerami P. Distinguishing epithelioid blue nevus from blue nevus like cutaneous melanoma metastasis using fluorescence in situ hybridization. *Am J Surg Pathol*. 2009;33:1396 - 400.
20. Siroy AE, Boland GM, Milton DR, Roszik J, Frankian S, Malke J, et al. Beyond BRAF(V600): clinical mutation panel testing by next-generation sequencing in advanced melanoma. *J Invest Dermatol*. 2015;135:508-15.
21. Boone B, Jacobs K, Ferdinande L, Taildeman J, Lambert J, Peeters M, et al. EGFR in melanoma: clinical significance and potential therapeutic target. *J Cutan Pathol.* 2011;38:492-502.
22. Sini MCD, V. Paliogiannis, P. Casula, M. Colombino, M. Manca, A. Botti, G. Ascierto, P.A. Lissia, A. Cossu, A. Palmieri, G. Genetic Alterations in main candidate genes during melanoma progression. *oncotarget.* 2018;9:8531-41.
23. Koroknai V, Szasz I, Hernandez-Vargas H, Fernandez-Jimenez N, Cuenin C, Herceg Z, et al. DNA hypermethylation is associated with invasive phenotype of malignant melanoma. *Experimental dermatology.* 2019.
24. Katunaric M, Jurisic D, Petkovic M, Grahovac M, Grahovac B, Zamolo G. EGFR and cyclin D1 in nodular melanoma: correlation with pathohistological parameters and overall survival. *Melanoma Res.* 2014;24:584-91.
25. Zhou R, Shi C, Tao W, Li J, Wu J, Han Y, et al. Analysis of Mucosal Melanoma Whole-Genome Landscapes Reveals Clinically Relevant Genomic Aberrations. Clinical cancer research : an official *journal of the American Association for Cancer Research*. 2019;25:3548-60.
26. Rakosy Z, Vizkeleti L, Ecsedi S, Begany A, Emri G, Adany R, et al. Characterization of 9p21 copy number alterations in human melanoma by fluorescence in situ hybridization. *Cancer Genet Cytogenet.* 2008;182:116-21.
27. Wang L, Rao M, Fang Y, Hameed M, Viale A, Busam K, et al. A genome-wide high-resolution array-CGH analysis of cutaneous melanoma and comparison of array-CGH to FISH in diagnostic evaluation. *J Mol Diagn.* 2013;15:581-91.
28. Gerami P, Scolyer RA, Xu X, Elder DE, Abraham RM, Fullen D, et al. Risk assessment for atypical spitzoid melanocytic neoplasms using FISH to identify chromosomal copy number aberrations. *Am J Surg Pathol.* 2013;37:676-84.
29. Glatz-Krieger K, Pache M, Tapia C, Fuchs A, Savic S, Glatz D, et al. Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization. *Virchows Arch.* 2006;449:328-33.