

FREQUENCY OF V600E MUTATION IN BRAF GENE AMONG PATIENTS WITH METASTATIC MELANOMA

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Abstract

This paper presents an overview of the frequency of V600E mutation in BRAF gene among patients with metastatic melanoma during the period from 2014 to 2018 at the University Clinical Centre in Banja Luka. Detection of mutations in BRAF gene was performed on 393 FFPE (formalin-fixed, paraffin-embedded tissue) skin tissue samples using the BRAF test through PCR (polymerase chain reaction) a standardized method for clinical analyses. Histopathological and molecular analyses of all samples were conducted at the Laboratory for Immunohistochemical and Molecular Diagnostics, Institute of Clinical Pathology, University Clinical Centre in Banja Luka. Study results clearly indicate that more than half of patients with metastatic melanoma carry V600E mutation in BRAF gene. Analysis confirms that high-quality quantity of DNA was successfully isolated from a large number of FFPE skin samples for mutation testing. Over the five-year study period, a statistically significant higher number of men than women were diagnosed with metastatic melanoma. Regarding the presence of mutations in the BRAF gene in relation to gender, frequency of BRAF+ and BRAF- samples was equal among men, while women had more BRAF+ samples compared to BRAF-. The average age of all patients with metastatic melanoma from the University Clinical Centre in Banja Luka was relatively low, only 58.62 years. The age group with the highest frequency of metastatic melanoma patients was between 55 and 65 years. BRAF V600E oncogenic mutations are among the most commonly identified molecular alterations in genomes of patients with metastatic melanoma and serve as the basis for selecting the best therapy. Detection of BRAF V600E mutations is an extremely applicable and widely used method that provides excellent results in clinical practice for selecting the optimal combination of cytostatic and treating metastatic melanoma today.

Key words: metastatic melanoma, molecular analysis, BRAF V600E mutation, gender and age categories of patients

INTRODUCTION

Skin is the largest organ of the human body and has numerous vital functions, such as protection from external influences (infectious, mechanical, thermal), maintaining body integrity, thermoregulation, absorption of ultraviolet (UV) radiation and detection of sensory stimuli (Curry *et al.*, 2012). Melanocytes in the skin epidermis are highly active cells as they synthesize and distribute pigment melanin. The name "melanocyte" originates from the Greek words where *melas* meaning dark and *cyte* meaning cell. They have dendritic extensions that extend from cell body between keratinocytes (Duncan, 2009). Pigment melanin is synthesized in endocytic vesicles of the melanocyte's endoplasmic reticulum, which are called melanosomes. Melanosomes contain enzymes responsible for melanin synthesis and transport it to surrounding keratinocytes, thereby protecting the skin from UV radiation (Roh *et al.*, 2017).

Metastatic melanoma (MM) is one of the most aggressive types of tumors in humans, ranking fifth in terms of frequency among all types of cancer worldwide. Mutations in the BRAF gene are observed in about 8% of human cancers, including about 60% of melanomas, 15% of thyroid tumors, about 12% of colorectal cancers, and 3% of lung cancers (Clarke *et al.*, 2017). In MM it was discovered that more than 97% of BRAF mutations are located within codon 600 of the BRAF gene, of which in about 90% of cases mutation is the result of transversion at the nucleotide. This transversion in the BRAF gene results in the replacement of valine with glutamic acid (Kong *et al.*, 2016).

In the process of malignancy development genes called oncogenes, play a significant role (Champan *et al.*, 2011). Oncogenes represent a modified form of normal genes, i.e., proto-oncogenes, whose primary function is to regulate cell growth and direct differentiation pathways through a process of signal transduction. This is a complex pathway of cellular messengers that leads from the cell membrane through the cytoplasm to the nucleus, where it participates in various types of information transfer to genes (Santiago *et al.*, 2015). Signal transmission begins with binding of RAS proteins and growth factors to a receptor on the cell membrane. This leads to a conformational change in RAS and its phosphorylation, resulting in activation of RAS. Activation of RAS proteins triggers a phosphorylation cascade in cytoplasm and leads to activation of RAF through RAS-RAF-MEK-ERK kinase pathway (Kraemer *et al.*, 1994). A mutated RAS gene causes continuous activation of RAS protein, promoting cell proliferation and inhibiting apoptosis. Most frequently mutated gene in this signaling pathway is the BRAF gene (Tsao and Niendorf, 2004).

MM characterized by V600E mutation is characterized by aggressive behaviour. Patients with this type of melanoma are generally younger, with metastases and a much shorter survival period compared to patients with melanoma without the mutation present (Longshore *et al.*, 2015). For these reasons, there is a need to determine presence of BRAF mutations in patients with MM, in order to choose optimal therapy. In accordance with new therapeutic possibilities that are aimed specifically at blocking the impact of mutation in tumors, such as, for example, BRAF inhibitors (Mahual *et al.*, 2017).

Relationship between age and gender of patients with MM and presence of BRAF gene mutation is correlated according to the literature. Therefore, aim of this study was to analyze and identify this potential correlation in a population of patients from University Clinical

Centre in Banja Luka. Results would greatly assist in future in prevention of MM in people of all age groups and in selecting best therapy for its treatment.

MATERIALS AND METHODS

Our study on the frequency of V600E BRAF gene mutation in patients with MM in University Clinical Centre in Banja Luka was conducted using parameters such as: the gender of patients; the concentration of isolated DNA from FFPET samples; success rate of BRAF V600E mutation detection; relationship between gender of patients with BRAF V600E mutation and presence of BRAF V600E mutation in patients of different age categories. Pathohistological and molecular analysis of all samples was conducted at the Laboratory for Immunohistochemistry and Molecular Diagnostics, Institute of Clinical Pathology at University Clinical Centre in Banja Luka. The analysis included data on detection of BRAF V600E mutation in a total of 393 FFPET skin samples from patients with MM over the period from 2014 to 2018. There was a total of 393 patients, of which 238 were men and 155 were women.

After the isolation was completed, concentration of obtained DNA was measured in order to confirm success of isolation, and to add appropriate required amount to the PCR mixture. The concentration of isolated DNA was determined on a Qubit®2.0 Fluorometer and Qubit dsDNA HS Assay kit of chemicals (ThermoFisher Scientific, USA). Assay kit contains a buffer for dissolving dye, a reagent with a fluorescent dye and two standards for calibrating the apparatus. Fluorescent dye binds to DNA and fluorescence intensity is proportional to DNA concentration. Amplification of BRAF gene segment and detection of BRAF mutations were performed using Cobas® BRAF V600 Mutation Test (Roche Diagnostics, Germany) according to manufacturer's instructions. Preparation of final volume of DNA involved volume of DNA isolate up to total required volume of 35 µl. Statistical analysis of obtained data included determination of the following parameters, and was done in standard Microsoft Excel for Office 365.

RESULTS AND DISCUSION

Detection of BRAF V600E mutation in formalin-fixed, paraffin-embedded skin samples of patients with metastatic melanoma is of critical importance, as it plays a key role in guiding therapeutic decisions. Our study demonstrated that a sufficient quantity of high-quality DNA was successfully isolated from a large number of samples, enabling reliable mutation testing. The clinical relevance of detecting this mutation extends beyond global medical practice, as it is also routinely applied in healthcare centres across Bosnia and Herzegovina.

This paper presents an analysis of the prevalence of BRAF V600E mutation in FFPET skin tissue samples from melanoma patients in metastatic stage. The number of FFPE tissue samples tested for this mutation at the University Clinical Center of the Republic of Srpska in Banja Luka increased steadily over the five-year study period (2014–2018). This trend reflects improvements in reliability and acceptance of this mutation detection method, as well as its expanding role in selecting optimal targeted therapies for patients with metastatic melanoma.

In our study on mutation detection in *BRAF* gene, real time PCR method was used, showing that more than half of metastatic melanomas have this mutation. Average prevalence of patients with metastatic melanoma at University Clinical Centre of Republic of Srpska in Banja Luka over three study years (2015, 2016, and 2017) was 27.83, while average incidence was 5.53. Among MM patients whose samples were tested there was a higher proportion of men likely due to occupational exposure to high-risk environments such as construction sites, metallurgy and mining.

Study achieved a high sample analysis success rate of 93.64%, ensuring reliability and validity of obtained results. BRAF V600E mutation assay was detected in 54.35% of FFPE skin tissue samples while it was absent in 45.65%. This distribution is consistent with existing literature, which reports similar mutation prevalence, with approximately half of samples exhibiting BRAF V600E mutation (Mourah *et al.*, 2015). Presence or absence of this mutation in FFPE skin samples suggests that MM can have both a genetic component (Swetter *et al.*, 2004) and an acquired nature, influenced by environmental and lifestyle factors (Tas and Erturk, 2017). Furthermore, among patients tested for the BRAF V600E mutation, men were more frequently affected than women (60.56% vs. 39.44%) (Figure 1). This gender disparity is statistically significant and may be attributed to increased exposure to risk factors such as chronic UV radiation, phenols, toxic fumes, ionizing radiation and heavy metals, particularly among older men with prolonged occupational exposure (Rigel, 2010). These factors are well-documented contributors to the pathogenesis of MM (Kim *et al.*, 2015).

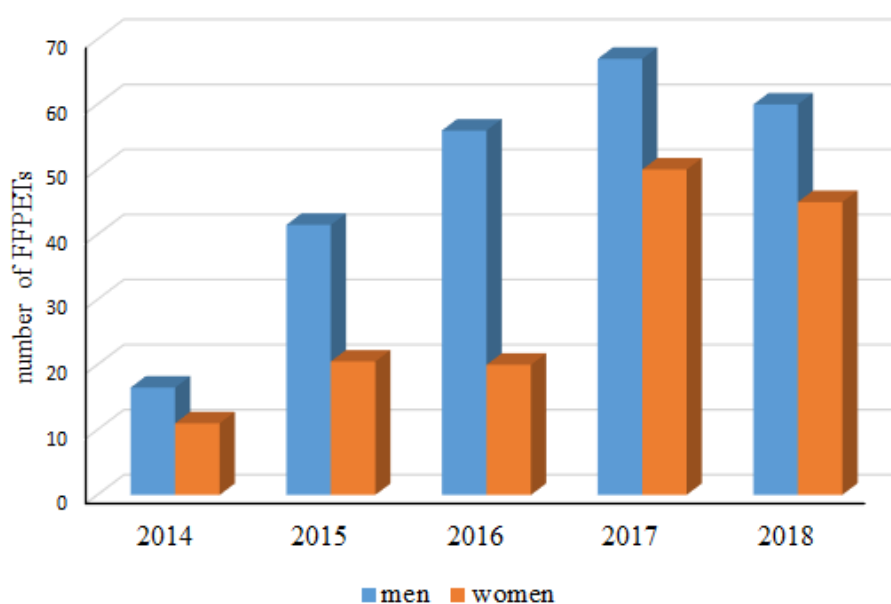


Figure 1. Presentation of number of FFPE samples in relation to gender of patients with MM cross study years of University Clinical Centre in Banja Luka

In terms of gender, there were 49.06% BRAF⁺ samples compared to 50.94% BRAF⁻ in males; while in females, there were 61.54% BRAF⁺ samples compared to 38.46% BRAF⁻. This data obtained in the study confirms the fact that men develop MM at a higher percentage due to worse working conditions rather than genetic predisposition. Average age of all patients with MM whose samples were tested was 58.62 years. This finding aligns with literature data, which

also confirm that MM predominantly affects older individuals due to aging, increased skin sensitivity and reduced ability of proper regeneration and repair (Cooper *et al.*, 2010).

Value of concentration of isolated DNA from FFPET samples appears to be very important for detection of BRAF V600E mutation. Standard value of the amount of DNA isolate, according to recommendations, should be equal to or greater than 5 ng/ μ l. In our study concentration of isolated DNA from FFPET samples was 89.62% for valid and 10.38% for invalid results, which indicates that the amount of isolated DNA was not the only cause of invalid results (Figure 2). Concentration and quality of isolated DNA are most important parameters for successful detection of BRAF V600 mutation in FFPET samples (Jurkowska *et al.*, 2015). A high-quality DNA sample is only valid parameter that will provide an accurate result and further therapeutic guidelines for the patient. (Pearlstein *et al.*, 2014; Malicherove *et al.*, 2018).

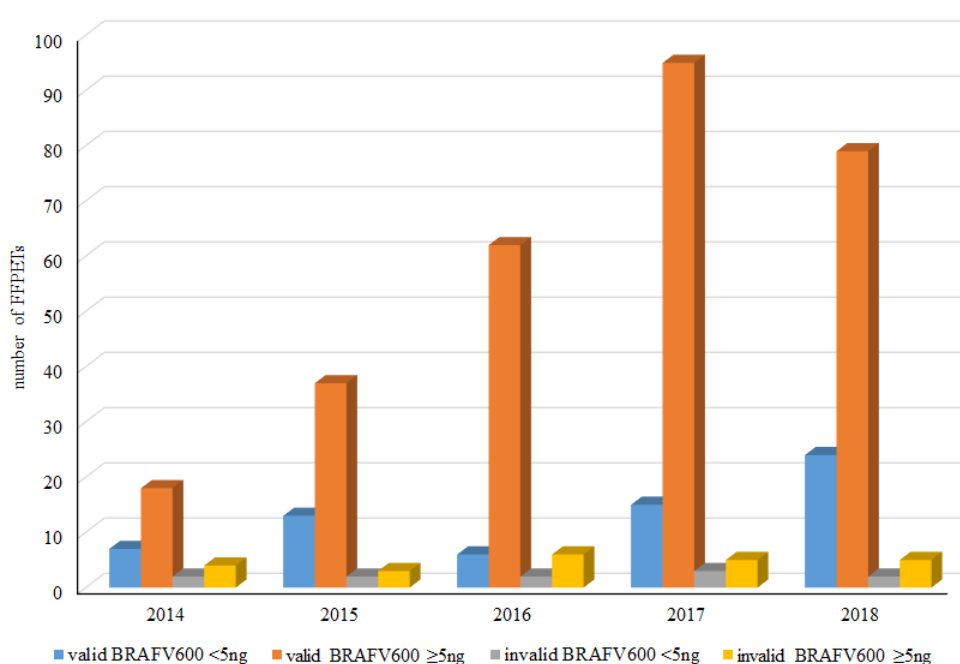


Figure 2. Distribution of tested FFPET samples according to amount of isolated DNA and validity of data in BRAF V600E mutations of University Clinical Centre in Banja Luka

In terms of gender (Figure 3), there were 49.06% BRAF⁺ samples compared to 50.94% BRAF⁻ in men while in women, there were 61.54% BRAF⁺ samples compared to 38.46% BRAF⁻. If the data are taken collectively, it can be seen that total prevalence of positive detection BRAF V600E mutation (49.06% or 104 samples) in FFPET skin tested samples of men suffering from MM is lower and approximates the percentage of samples without detection (50.94% or 108 samples). On other hand, in the case of samples from women suffering from MM (156), percentage of positive detection of BRAF V600E mutation is significantly higher and amounts to 61.54% (or 96 samples) compared to 38.46% (or 60 samples) of women in whom no mutation was detected.

In our study, there are definitely more male patients affected by MM compared to females, which is not the case with authors publishing studies in the same field. According to some, number of affected men and women is approximately equal (Wu *et al.*, 2017), while

according to others, there are more affected women (Roh *et al.*, 2017). An interesting finding in this study is high percentage of men who develop MM but do not have mutation in BRAF V600 gene, which suggests that they acquired disease after several decades of life. These data also differ from literature, which confirms that number of patients with BRAF+ and BRAF- mutations is roughly equal, regardless of gender (Anderson *et al.*, 2012; Chat-Uthai *et al.*, 2018).

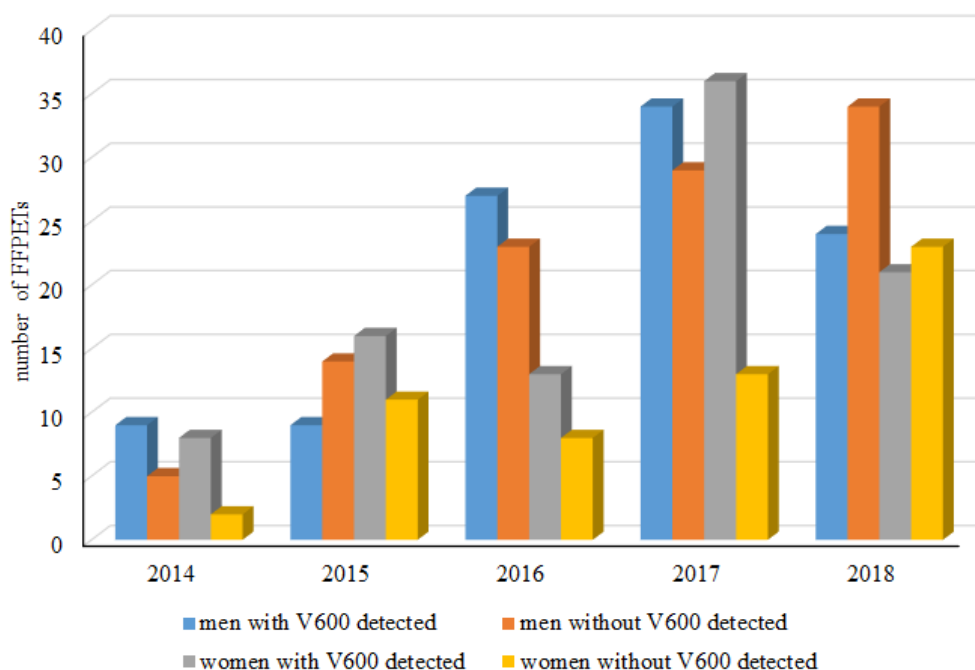


Figure 3. Representation of frequency of detection V600E mutation in BRAF gene in relation to gender of patients of University Clinical Centre in Banja Luka for period from 2014 to 2018

The average age of all patients with MM whose samples were tested was 58.62 years. In our study, the age category with highest number of patients at 27.45% is between 55 and 65 years old, and majority number of them are men. The majority of patients with MM whose FFPET samples were tested for BRAF V600E of University Clinical Centre in Banja Luka were from age category 55-65 in 2015 as well as in 2016 and 2017, while most patients were from age category 35- 45 in 2014 and from 45-55 category in 2018 (Figure 4). Second age category in terms of number of patients is 45-55, followed by 65-75. In 2017, a case of a person suffering from metastatic melanoma from age category 1-15 was recorded. More precisely, it is about a patient who is only three years old, a boy who is BRAF V600E positive. Likewise, in 2016 and 2017, two patients each from the 15-25 age groups were recorded, for a total of three male and one female patients. Distribution of patients with MM according to age is expected and aligns with the literature cited by other authors, according to majority of literature sources, that disease occurs more frequently in older individuals (Swetter *et al.*, 2004; Tsao and Niendorf, 2004).

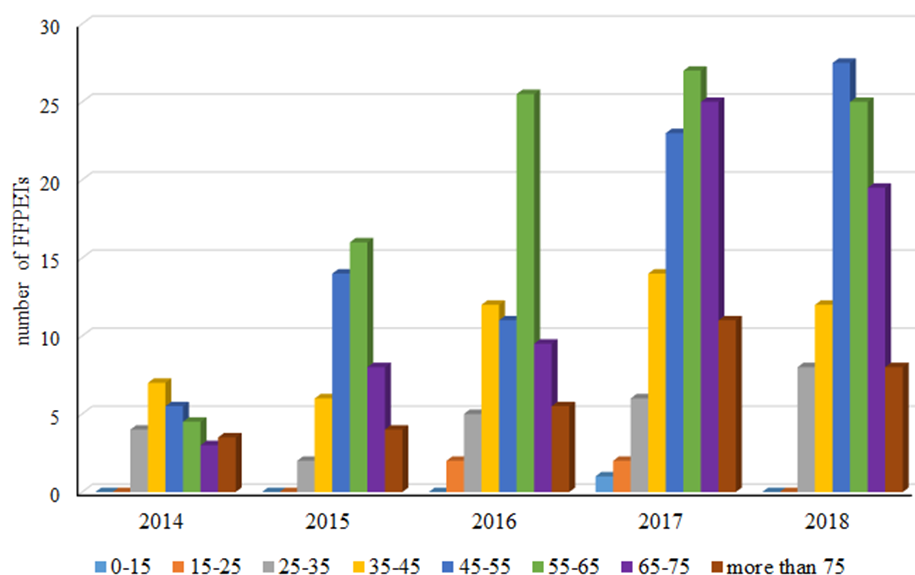


Figure 4. Frequency of tested FFPE samples in relation to age categories of patients with MM by year of University Clinical Centre in Banja Luka for period from 2014 to 2018

According to the majority of literature sources, patients who develop MM are typically in the age category over 60-70 years (Pearlstein *et al.*, 2014; O'Brien *et al.*, 2017). As seen in our study, the age threshold for developing MM has shifted upwards. Authors of other studies also noted that threshold for developing MM was moving upwards, as younger individuals were increasingly affected by this disease (Tas and Erturk, 2017; Zocco *et al.*, 2020). The reason for this shift can be found in changes in lifestyle and habits of modern humans, as well as climate changes occurring on our planet (Rigel, 2010; Qu *et al.*, 2013).

CONCLUSIONS

The BRAF V600E oncogene mutations are the most commonly detected parameter in the diagnosis of melanoma of all tissue types including skin. Detection of these mutations is crucial for guiding and selecting optimal therapies for treatment of metastatic melanoma. The use of BRAF gene mutation detection is widely applicable in global medical practice, as well as in healthcare centers in University Clinical Centre in Banja Luka. However, in addition to its applicability, this study also presents frequency of mutation occurrence in relation to gender and age of patients. The study showed that average age of all patients was below expected average, with a higher incidence of male patients who did not have BRAF gene mutation. Results of study indicate that patients, whose FFPE skin samples were tested, most frequently did not have a genetic predisposition to develop metastatic melanoma, but rather, the disease was influenced by environmental factors.

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ФРЕКВЕНЦИЈА В600Е МУТАЦИЈА У БРАФ ГЕНУ КОД ПАЦИЈЕНАТА СА МЕТАСТАТСКИМ МЕЛАНОМОМ

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Сажетак

Рад представља приказ фреквенције мутације В600Е у БРАФ гену код пацијената обољелих од метастатског меланом у периоду од 2014. до 2018. године на Универзитетском клиничком центру у Бања Луци. Детекција мутација на БРАФ гену вршена је из 393 ФФПУТ (формалин-фиксираним, парафин-укалуљеним ткивима) узорака коже помоћу БРАФ теста преко ПСР (ланчана реакција полимеразом) стандардизоване методе за клиничке анализе. Патохистолошка и молекуларна анализа свих узорака спроведена је у Лабораторији за имунохистохемијску и молекуларну дијагностику, Института за клиничку патологију Универзитетског клиничког центра у Бања Луци. Резултати студије јасно показују да В600Е мутацију у БРАФ гену има више од половине пацијената са метастатским меланомом. Анализа резултата доказује да је из великог броја ФФПУТ узорака успјешно изолована квалитетна количина ДНК за тестирање мутација. Током петогодишњег периода обухваћеног студијом било је статистички значајно више обољелих мушкараца него жена са метастатским меланомом. Присуство мутација у БРАФ гену у односу на пол била је једнака фреквенцији БРАФ+ и БРАФ- узорака код мушкараца, док је код жена било више БРАФ+ узорака у поређењу са БРАФ-. Просјечна старост свих пацијената са метастатским меланомом са Универзитетског клиничког центра у Бања Луци била је релативно ниска, свега 58,62 године. Старосна категорија са највећом фреквенцијом пацијената са метастатским меланомом била је између 55 и 65 година. БРАФ В600Е онкогене мутације су најчешће коришћене молекуларне промјене у геномима пацијената са метастатским меланомом и служе као основа за одабир најбоље терапије. Детекција БРАФ В600Е мутација је изузетно примјенљива и широко коришћена метода која пружа одличне резултате у клиничкој пракси за одабир комбинације цитостатика и лијечење метастатског меланом данас.

Кључне ријечи: метастатски меланом, молекуларна анализа, БРАФ В600Е, пол и старост пацијената

Received March 03, 2025

Accepted April 04, 2025