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The BRAF V600E Mutation Detection by quasa Sensitive Real-Time PCR Assay in Northeast Romania Melanoma Patients

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Abstract: Background: The prevalence of melanoma in Romanian patients is underestimated. There is a need to identify the BRAF V600E mutation to accurately treat patients with the newest approved BRAF inhibitor therapy. This is a pilot study in which we first aimed to choose the optimal DNA purification method from formalin fixation and paraffin embedding (FFPE) malignant melanoma skin samples to assess the BRAF mutation prevalence and correlate it with clinical pathological parameters. Methods: 30 FFPE samples were purified in parallel with two DNA extraction kits, a manual and a semi-automated kit. The extracted DNA in pure and optimum quantity was tested for the BRAF V600E mutation using the quantitative allele-specific amplification (quasa) method. quasa is a method for the sensitive detection of mutations that may be present in clinical samples at low levels. Results: The BRAF V600E mutation was detected in 60% (18/30) samples in patients with primary cutaneous melanoma of the skin. BRAFV600E mutation was equally distributed by gender and was associated with age >60, nodular melanoma, and trunk localization. Conclusions: The high prevalence of BRAF V600E mutations in our study group raises awareness for improvements to the national reporting system and initiation of the target therapy for patients with malignant melanoma of the skin.

Keywords: melanoma; BRAF V600E; mutation; frequency; target therapy

Citation: Porumb-Andrese, E.; Ursu, R.G.; Ivanov, I.; Caruntu, I.-D.; Porumb, V.; Ferariu, D.; Damian, C.; Ciobanu, D.; Terinte, C.; Iancu, L.S. The BRAF V600E Mutation Detection by quasa Sensitive Real-Time PCR Assay in Northeast Romania Melanoma Patients. *Appl. Sci.* **2021**, *11*, 9511. https://doi.org/10.3390/app11209511

Academic Editors: Francisco Arrebola and David Mills

Received: 6 August 2021
Accepted: 11 October 2021
Published: 13 October 2021

1. Introduction

Melanoma is the most aggressive form of skin cancer that can arise from a preexisting nevus or on normal skin. The estimated annual increase in incidence is 3–7% worldwide for Caucasians. Higher rates are recorded in countries with higher exposure to UV radiation at low latitudes, such as Australia, Scotland and England. From this perspective, Europe may be considered an exception, as a higher incidence of melanoma is recorded in such countries as Denmark, Sweden and Norway while a low incidence is reported in Greece or Bulgaria [1,2].

According to GLOBOCAN 2020, the estimated crude incidence rates in 2020 of melanoma of skin, for both genders, ages 0–74 in Romania was 7.2, while Nordic countries were registered as having higher values: Denmark with 41.9, The Netherlands with 40.6 and Norway with 36.7 [3].

The rating appears to be a result of significantly underestimating the incidence at the national level. Although, in Romania, a National Cancer Registry exists, the melanoma data are not complete, due to a lack of rigor in transmitting this information. Usually, the diagnosis is made by various specialists, so the responsibility of communication is divided; therefore, oncological disease requires a complex multidisciplinary approach.

A recent survey carried out in melanoma centers from 27 European countries mentioned the important role of dermatologists highly trained in dermatoscopy, dermatosurgery, follow-up and the systemic treatment of melanoma and melanoma detection [4].

The unanimously recognized risk factors of melanoma are a high number of nevi (higher risk if nevi are dysplastic), family history of melanoma, immunosuppressive state, skin phototype I or II, Dubreuillh melanosis and history of Non-Melanoma Skin Cancer (NMSC) [5].

The mutation that causes a valine (V) to glutamic acid (E) substitution at codon 600 is named BRAF V600E. BRAF inhibitors are used for melanoma therapy, such as vemurafenib, the first specific inhibitor acting on the BRAF/MEK pathway approved by the Food and Drug Administration, together with vemurafenib, dabrafenib and encorafenib, which also belong to a group of drugs called BRAF protein inhibitors. A significant impact was demonstrated on patients with melanoma and the BRAF V600E mutation [6]. Rapid progress is being made in the determination of potential new targets, mechanisms of resistance and development of additional rational therapies [7,8].

There are a few methods for BRAF detection, ranging from immunohistochemistry to hybridizations on strips, array analysis and real-time PCR sequencing. Each method has advantages and disadvantages. Molecular oncologic testing is an important step in the diagnosis and treatment of many types of skin cancer. Formalin fixation and paraffin embedding (FFPE) is the gold standard for the long-term preservation of most archived skin cancer specimens. These samples represent the best resource for subsequent molecular studies of clinical phenotypes. Genetic studies are the main type of research, in which the obtained DNA is used for a variety of tests. FFPE usefulness is underlined by the fact that, often, access to fresh tissue can be difficult and studies on this type of material can be achieved at any time, from diagnosis or even post-mortem. Isolating DNA from FFPE is a basic condition in achievement of the molecular testing and there are some challenges in obtaining the optimal quality and quantity: first, in the tumor cell availability in these specimens and, second, in the possible presence of the contamination of tumor cells with necrotic or inflammatory cells or other non-neoplastic cells [9].

At present, almost 70 commercial kits are available, from which 35 were developed specifically for DNA extraction. The tissue deparaffinization step is omitted in many protocols by melting paraffin directly into a tissue lysis buffer. It would be useful for researchers to use commercially available kits for the purification of DNA from FFPE, instead of old-fashioned, crude and probably less effective in-house methods [10].

Hypothesis and Aims

1. To assess, for the first time in Romania, the proportion of BRAF V600E mutation using quantitative allele-specific amplification (quasa) in melanoma FFPE samples. 2. to correlate the BRAF mutation with possible risk factors—age, gender and melanoma localization—in order to predict the prognostic and efficiency of future target treatment.

2. Materials and Methods

2.1. DNA Purification of FFPE Samples

The FFPE melanoma samples were selected from those registered from the years 2013–2014 at the Regional Oncologic Institute and "St. Spiridon" Hospital, Iaşi. Through a

retrospective analysis, we collected clinical data such as gender, age, site of tumor, TNM stage, Breslow thickness or Clark level. Because the amount of DNA extracted and its quality depend on several factors, including the period of paraffin embedding or storage conditions, we only used blocks recently embedded in paraffin (2013–2014) to reduce the risk of degradation of the genetic material. We excluded melanomas with a location other than the skin, metastases, and patients with few FFPE (where the amount of archived material would not have legally allowed access to tumor tissue) from the analysis and formed our study group from the remaining samples. The blocks were selected by a pathologist experienced in oncology and sections were made after marking the area of interest. Thermo Scientific™ DNA AWAY™ Surface Decontaminant solution between samples was used to avoid contamination. From each sample, four sections were realized, of 10 µm each, with two sections per tube, which we used in their entirety in the DNA extraction. We evaluated two DNA FFPE extraction kits in parallel for each sample: a manual (PureLink® Genomic DNA Kits/Life Science) and a semi-automated kit (Innu PREP FFPE DNA KIT/Analitik Jena). The DNA quantity and purity were measured with Thermo Scientific NanoDrop ND-2000c.

2.2. quasa Experiments

We used the BRAF V600E mutation detection kit by quantitative allele-specific amplification (quasa) kit/Primer Design and MX3005P Stratagene thermocycler. quasa is a method for the sensitive detection of mutations that may be present in clinical samples at low levels [11]. This method is based on "allele-specific PCR", using modified primers and a hydrolysis probe, modified cycling conditions and a modified mastermix. In the quasa method, the 3′ terminal base of the mutation-detecting primer is sited to bind to the mutant base. In the samples where the mutation is present, efficient amplification results in detection of the mutant sequence, whereas PCR, from this primer, is blocked on wild-type samples due to the mismatch. quasa primers are designed such that the 3′ terminal base overlies the mutation site. Thus, wild-type primers confer 100% specificity with the wild-type sequence but have a single base mismatch with the mutated sequences. This is typical of allele-specific PCR and relies on the principle that the single base mismatch will prevent the wild-type primers from successfully priming on the mutated template. The quasa protocol uses a two-stage cycling strategy. The first ten cycles of PCR used a very low annealing temperature of 50 °C. This allows the low Tm primers to prime successfully whilst conferring the highest possible levels of specificity. After the first ten cycles, the annealing temperate is switched to 60 °C. Thus, allele-specific priming is effectively blocked and priming only occurs where the tagged primers have been incorporated. This also drives highly efficient amplification and probe cleavage and thus confers the sensitivity of the method [12].

3. Results

3.1. Clinical Findings

Most of the patients were from the rural area (56% versus 43%) and, at the time of diagnosis, the mean age of patients was 58.8 (range, 36 to 81 years) (Table 1). In the total group, 14/30 patients (46.6%, respectively) were women; the most frequent localization of melanoma was on the chest (16/30, 53.3%), followed by limbs (11/30, 36.6%); and another 3 patients had different melanoma localizations. Regarding the histological category, 13/30 patients (43.3%) were registered with nodular melanoma, the same percentage (43.3%) with superficial spreading melanoma and, for 4 of the samples, the histological form was unknown. Clark level (the tumor invasion through the layers of the skin) was available only for 24 samples: 2 samples had level II, 7 III level, 12 level IV and 3 had level V.

Table 1. Descriptive statistical indicators of age (years) by sex.

Sex	No.	Media	Standard Deviation	Standard Error	Confidence Intervals		Min	Max
					Lower Limit	Upper Limit		
Females	14	58.86	12.97	3.47	51.37	66.34	36	88
Males	16	58.81	17.06	4.27	49.72	67.9	25	81
Total	30	58.83	15.03	2.74	53.22	64.45	25	88

All the statistical analyses were performed using SPSS version 24.0 software (IBM Corp., Armonk, NY, USA). The normality of data was assessed using skewness and kurtosis tests and a visual inspection of histograms. Student's *t*-test was applied for a comparison of the means of normally distributed series of values, depending on the number of degrees of freedom (df). All means and medians were reported with appropriately calculated 95% confidence intervals. Associations between the categorical variables were assessed by Chi-square tests which were applied in two study groups for excluded events.

Synthesizing the demographic characteristics of the study group, the preponderance of patients over 60 years of age, male and from rural areas is noticeable. In the cases studied, the location on the chest (56.7%) and limbs (33.3%) predominates and, in three patients (10%), the origin of melanoma was different (scalp or retroauricular).

3.2. DNA Purification from FFPE Melanoma Samples

The manual kit used xylene for paraffin extraction and involved one 3-h-long incubation. The semi-automated kit needed just 1 h of incubation and then the DNA extraction was performed automatically in 37 min by InnuPure® C16 equipment. Using the manual kit, we obtained a very low quantity of DNA with eight of them having too low a DNA quantity, which were consequently unable to be detected by the nanodrop. With this DNA, we were not able to amplify the patients' samples for both BRAF V600E and wild type. The positive controls were present each time, at the correct Ct values (24–32). With the semi-automated kit, we obtained a high amount of pure DNA from the same samples, with two sections per tube. The 260/280 ratio was between 1.68 and 2.08. In all the positive cases, we obtained amplification for both BRAF primers—V600E and wild type.

3.3. QuASAR BRAF V600E Detection

Our real-time PCR quasa experiments were validated by using negative and positive controls. From the tested samples, we detected 18/30 as positive for BRAFV600E. The percentage of single nucleotide polymorphisms (SNPs) present in the sample was calculated using the delta Cq method. The proportion of SNP in the sample was then corrected by reference to the standard, which is present at a known proportion of 1%. The calculation was performed in three stages. Firstly, the delta Cq values were used to calculate relative detection levels between wild-type and mutant sequences for both the biological samples and the 1% control sample. This was then converted into a percentage. The 1% sample was then used as a normalizing signal for the biological data by creating a K value that corrects any variation between samples and machine settings. We used the next equations, suggested by quasa insert (Delta Cq calculation = 2ˆ-(BRAF wild-type-BRAF V600E); Percentage conversion = 1/Delta Cq *100.

4. Discussion

4.1. DNA Extraction Methods

Large differences between the two kits in terms of the amount of DNA obtained from the samples were observed. The omission of dewaxing from the reaction appears to protect paraffinized DNA strands in FFPE tissue from rapid degradation during the extraction process. The semi-automated kit was the optimum solution for DNA extraction from our selected FFPE skin samples, as the semi-automated kit had all the advantages of automatization (possibility of processing 16 samples in almost 40 min, no cross-contamination,

highly reproducible); the most important feature for our samples was the isolation of very pure nucleic acids in adjustable elution volumes.

The results of Mourah S. et al. [13], regarding DNA elution volume between manually and automated extraction, are in line with our findings. Our results suggest that the archival FFPE skin tissues had an important genetic value, and the quantity of the extracted DNA depends on the optimal solution being chosen from the multitude of commercial kits available. Our study also confirms the importance of choosing the most reliable and constant FFPE DNA extraction system for sample preparation, especially when using small biopsies and low elution volumes in routine diagnostics [14,15].

4.2. Proportion of BRAF V600E

We detected a 60% prevalence of BRAF V600E mutation in our study group, which can be compared with the results of other studies carried out in the European population: 28.6% (12/42) in Hungary, 32.3% (136/420) in France and 57% (916/28) in Denmark [16–18]. It can be observed that the allele-specific PCR (TaqMan), which also involves mutation detection status using a predetermined cut-off Ct value method, gave a high proportion of BRAF mutations, as in our study group. As there are no available guidelines for choosing the molecular techniques used for BRAF mutation detection, as in the case of other cancers (e.g., cervical cancer), we should choose the most sensitive method, to ensure the highest chance of detecting all positive patients.

In the future, it would be useful to confirm our data with a second method, such as immunohistochemistry, and on a larger study of group patients [19].

The molecular assays are continuously developing, and it is now possible to directly test for BRAF V600E mutation from the plasma of melanoma patients, which will avoid the difficulties of FFPE sample processing [20].

4.3. BRAF V600E Mutation Association with Clinical Data

Regarding the relationship between the presence of BRAF V600E mutations and various environmental factors, gender, or anatomo-pathological features, we found the following: the gender of patients could not be correlated with the presence of mutations (9 female/9 male and the same result for the area of residence). The mean age at the time of mutation occurrence was 59 years (8 patients $\leq$ 60 years and 10 patients > 60 years), identical to the mean age of patients without mutation. The mutations were more common in lesions located on the trunk (10 out of 18).

Regarding the histopathological findings, there are studies that failed to find any statistical correlation between them and BRAFV600E [21]. In our case, the mutation was more frequent in lesions invading the reticular dermis (Clark level IV). Since some studies support a correlation between mutation rate and the histological form of melanoma (i.e., a higher frequency in nodular and superficial spreading melanomas versus acral melanomas) [22], this could explain the high mutation rate found by us as, of the 18 positive cases, only one patient presented an acral form. It was also found that the mutation is more common among Caucasians than Asians, with a mutation rate of about 23% in the Chinese population, a fact that could explain our result [23]. A lower frequency of mutations was found in non-cutaneous tumors (mucosal and uveal location) or ungual melanomas, a fact also supporting our results because all samples included in the study were of cutaneous melanomas [24]. However, there are authors who established an inverse correlation between the presence of BRAFV600E mutation and exposure to UV radiation. A more frequent identification of this mutation among lesions on non-exposed areas is confirmed in our study by the fact that 10/18 were represented by cutaneous melanomas on the trunk [25]. For the analyzed cases, we found the next association between Clark level and BRAFV600E mutation: 40% were level IV, 10% level V and 16.7% II or III level.

4.4. Clinical Utility of BRAF V600E Detection

In 2011, a clinical trial conducted on 675 patients with previously untreated, metastatic melanoma with the BRAFV600E mutation concluded that the BRAF kinase inhibitor vemurafenib produced improved rates of overall and progression-free survival in this category of patients [17]. In an extended follow-up analysis of patients older than 18 years, with treatment-naive metastatic melanoma and whose tumor tissue was positive for BRAF(V600) mutations, it was also observed that an inhibition of BRAF with vemurafenib improved survival [20]. A retrospective study revealed that vemurafenib appears to have predictable and manageable adverse events (rash, squamoproliferative growths, photosensitivity and squamous cell carcinoma or keratoacanthoma). Proactive management can limit the impact of adverse effects (AEs) on patients, allowing for treatment to continue despite toxicities [26].

The therapeutic benefits of combination of vemurafenib and IFN-α in patients with BRAFV600-mutated melanoma were found in a recent 2021 study [27].

All these factors suggest that we need a more optimized screening of melanoma of the skin in our country, using molecular methods. The targeted therapy is also new for our area, but by proving the presence of BRAF mutations, we may advise the use of these new drugs.

A limitation of the study was the limited number of melanoma cancers that were analyzed. This was mainly due to the fact that only the analyzed archival melanoma cancers were available in the moment of FFPE selection from the hospital archive. The results of this study warrant the implementation of BRAF testing as routine in the case of melanoma patients.

In conclusion, we detected a 60% prevalence of BRAF V600E mutations in this pilot study group of melanoma patients using a sensitive, accurate and fast QuASAR real-time PCR method. These data can be used for the selection of patients with melanoma, as candidates for target therapy.

Author Contributions: E.P.-A., R.G.U., and L.S.I. were responsible for the research design. V.P., I.-D.C., D.C., C.D. and D.F. resources, original draft preparation, I.I., C.T. and D.C. composed and analyzed the datasets. All authors have read and agreed to the published version of the manuscript.

Funding: This work was developed within the project "Program of excellence in multidisciplinary doctoral and postdoctoral research regarding chronic diseases" (POSDRU 159/1.5/S/133377), Grigore T. Popa University of Medicine and Pharmacy, Iași.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data and materials supporting the results of the present study are available in the published article.

Acknowledgments: R.G. Ursu and C. Damian are supported by "Grigore T. Popa" University of Medicine and Pharmacy Iași (research contract 6983 from 21.04.2020).

Conflicts of Interest: The authors declare no conflict of interest.

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