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ORIGINAL ARTICLE

Investigating EGFR, BRAF, and RAS Mutations in Oral and Cutaneous Squamous Cell Neoplasms: A Preliminary Report on Romanian Patients

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Abstract: *Background: Head and neck cancers, and particularly, oral cancers have a complex pathogenesis that includes genetic mutations and epigenetic alterations which interfere with cellular signaling and can trigger tumor development. The purpose of this study was to reveal whether low-frequency hotspot mutations may be detected in a study lot with histopathological evidence of squamous cell carcinoma (SCC) of the oral mucosa and skin of the head and neck. Methods: Tumor biopsies from treatment-naïve patients were tested for BRAF V600, NRAS G12/G13, NRAS Q61, KRAS Q61 mutations, and EGFR exon 19 deletions (Ex19Del) using droplet digital PCR (ddPCR). The tumors were also analyzed for EGFR T790M mutations by RT-PCR, using a CE-IVD validated kit, with a limit of detection of 0.05%. Results: None of the examined cases exhibited NRAS G12/G13, NRAS Q61, KRAS Q61, BRAF V600, or EGFR T790M mutations, indicating that these alterations are rare events in SCC pathogenesis. Interestingly, among the 12 specimens tested by ddPCR for EGFR Ex19Del, an HPV-negative cSCC tumor occurring in the parotid region tested positive for this drug-sensitizing mutation, offering unexplored therapeutic perspectives to the patient from whom it was collected. Conclusions: Our study highlights the important clinical implications of detecting low-frequency hotspot mutations in tumor biopsies by ddPCR. We believe that the ddPCR-assisted analysis of these mutations in larger SCC cohorts may provide us with mechanistic insights regarding their role in SCC pathogenesis and guide the development of novel therapeutic strategies for this problematic disease.*

Keywords: *head and neck squamous cell carcinoma; droplet digital PCR (ddPCR); targeted therapy, immunotherapy*

INTRODUCTION

Squamous cell carcinomas (SCCs) are one of the most unpredictable and widespread neoplasms globally, presenting a significant socio-economic burden on healthcare systems worldwide [1]. SCC development has been linked to the dysregulation of several molecules involved in different stages of squamous lineage commitment and keratinocyte terminal differentiation [2]. However, the exact identity and the precise order of the genetic and epigenetic alterations facilitating keratinocyte malignant transformation remain elusive. Several research groups postulated that epidermal clones with mutated p53 may be at the origin of SCC, as they lead to squamous dysplasia and in situ SCC, which subsequently progress to metastatic SCC [3]. At the same time, emerging evidence suggests that the disruption of the mitogen-activated protein kinase (MAPK) signaling pathway, activated by epidermal growth factor receptor (EGFR), facilitates the development and progression of various malignancies, including head and neck squamous cell carcinoma (HNSCC) and cutaneous SCC (cSCC) [3,4]. The MAPK pathway may be depicted as a complex regulatory network that links the tumor microenvironment with the cell nucleus, converting the

extracellular signals into intracellular responses such as cell proliferation, differentiation, or apoptosis [4,5]. RAFs (A-/B-/C-RAF), RASs (N-/H-/K-RAS), MEKs, and MAPKs kinases are the most notorious constituents of the MAPK signaling pathway, behaving as powerful oncogenes in various human cancers. Thus, any alteration within those signaling components may lead to apoptosis resistance and uncontrolled cellular growth [4]. Beyond the critical role of EGFR/MAPK signaling in HNSCC development, the various nodal points within the signaling pathway can be converted into therapeutic vulnerabilities in these neoplasms. For instance, BRAF V600E-mutant ameloblastomas may be targeted with BRAF inhibitors such as vemurafenib and dabrafenib, whereas HRAS-mutant HNSCC can be approached with farnesyltransferase inhibitors (FTis) like tipifarnib [4,6]. Despite these recent discoveries, the main therapeutic approach for HNSCC and cSCC combines surgery with radiation therapy and systemic chemotherapy, with ancillary reconstructions depending on case complexity and structures involved [7–9]. Surgical excision of the tumor mass remains the cornerstone of treatment for resectable SCC, followed by adjuvant radiotherapy, with or without chemotherapy [10,11]. The tumoral resection may carry additional risks when considering local infiltration, anatomical variants, or surgical approach [12–14].

The stratified epithelium from which SCC originates can be found distributed on the surface of the outer integument or covering organs and cavities such as the oral cavity, sinonasal cavity, oropharynx, larynx, esophagus, lung, and anogenital region [15]. SCCs are currently classified and examined taking into account the anatomical divisions of the human body; therefore, the SCCs of the head and neck are treated differently from those of the lung [2]. Here, we will focus exclusively on SCCs of the skin and of the mucosal epithelium of the oral cavity and lips (OSCC). Although both tumor types arise from the disruption of the same differentiation programs, cSCC and OSCC are considered distinct entities with different risk factors [16,17]. The main etiological factors for OSCC include tobacco and alcohol usage, and human papillomavirus (HPV) infection [15,18,19]. For cSCC and lower lip SCC, the most prominent risk factor remains increased exposure to ultraviolet radiation (UVR), followed by a personal history of skin cancer and HPV infection [19,20].

cSCC is the second most common cancer worldwide, and its incidence is steadily increasing in the Caucasian population. As cSCC is often invasive, inaesthetic, and locally destructive, such diagnosis usually involves complex skin surgeries and facial reconstructions [21]. While the majority of cSCC patients present with a very good prognosis, 1.5-5.2% of cases may develop life-threatening metastases [22,23]. Moreover, owing to the high incidence rates, in the United States, the mortality rate associated with cSCC is comparable to that reported for more aggressive tumors such as leukemia and melanoma [22]. The number of cSCC-associated deaths is hence expected to surpass that of melanoma in the next 5 years, mainly due to the rise in HPV infections [24]. HNSCC, the sixth most common type of cancer globally, represented mainly by OSCC, also exhibits a poor overall survival (OS) rate. Despite advancements in diagnostic and treatment methods, the HNSCC 5-year OS rate remains approximately 50% [25], suggesting that more intensive scientific efforts should be devoted to understanding the complex biology of HNSCC tumors. Molecular studies to identify the key factors driving cSCC and OSCC development are ongoing, aiming to identify the most promising diagnostic biomarkers and therapeutic targets for these problematic diseases [5,26,27].

The latest advancements in omics technologies facilitated the identification of several hotspot mutations in the EGFR/MAPK signaling pathway which have been harnessed for targeted pharmacological interventions in cancer patients. For instance, the observation that 80% of HNSCCs exhibit EGFR overexpression led to the development and subsequent approval by the Food and Drug Administration (FDA) of Cetuximab, an anti-EGFR monoclonal antibody, to be used in combination with radiotherapy in locally advanced HNSCC [28]. Yet, the dissection of the mutational spectrum of the EGFR gene served as a foundation for the development and implementation of novel EGFR-targeted therapies in clinical settings. For instance, genetic alterations such as exon 19 deletions (Ex19Del) and exon 21 substitutions (e.g., L858R), representing the most common EGFR mutations in non-small cell lung cancer (NSCLC), confer sensitivity to first (gefitinib and erlotinib) and second-generation (nilotinib, dasatinib) tyrosine kinase inhibitors (TKIs) [29]. In addition, exon 20 substitutions which include EGFR T790M mutations and are generally associated with NSCLC resistance to first-generation TKIs, may be also matched with targeted therapy in cancer patients, particularly with third-generation TKIs such as osimertinib and alflutinib [29]. In HNSCC/cSCC clinical cohorts, single-target TKIs, such as gefitinib and erlotinib were shown to improve modestly patient survival rates when given as monotherapy [30]. However, promising results have been reported when small TKIs were combined with other agents such as pembrolizumab, cisplatin, or docetaxel, in recurrent and metastatic settings [31]. This suggests that EGFR mutations may be exploited for targeted therapeutic interventions in the future, once the clinicians validate the most beneficial drug combinations. Alternative strategies for tailored therapy in HNSCC include a) FTIs, such as tipifarnib, for HRAS-mutant HNSCC (recently FDA-approved); b) cetuximab for SCC presenting with KRAS germline variants; c) EGFR inhibitors for HNSCC tumors harboring MAPK1 mutations; d) BRAFi and

MEKi for BRAF V600E-mutant ameloblastomas, combined or not with immunotherapies [4].

As we described earlier, the genetic assessment of BRAF, a serine/threonine protein kinase mutated in 60% of cutaneous melanomas and more than 15% of human cancers, may also expand the anti-cancer armamentarium in cSCC/HNSCC [32]. Despite the low prevalence of BRAF mutations in SCC of the head and neck (2%), HNSCC patients carrying these alterations present with improved clinical outcomes after BRAF and MEK inhibition [33]. RAS is another key oncogene in the pathogenesis of human cancers. KRAS mutations were identified in 2.89% of HNSCC cases, most frequently in tumors with sinonasal localization, whereas NRAS alterations were reported in 2.2% of cases, especially in tumors of the nasopharynx [34]. A recent meta-analysis highlights that oncogenic KRAS may be associated with significantly enhanced immune checkpoint molecule (ICM) expression and immune evasion programs within the tumor microenvironment, making KRAS-mutant tumors promising candidates for immune checkpoint inhibition (ICI) in the clinical setting [35]. However, several recent reports highlight that the MAPK-mutant status may serve as an indicator of a favorable response to immunotherapy in HNSCC, mainly due to the increased CD8+ T cell and dendritic cell infiltrations reported within these tumors, representative of an active cytolytic CD8+T-inflamed status [36].

The present study aims to explore the role and prevalence of KRAS, NRAS, BRAF, and EGFR mutations in a pilot group of patients diagnosed with SCC of the oral mucosa and skin.

MATERIALS AND METHODS

Patients and samples

Twelve patients diagnosed with squamous cell neoplasms of the head and neck between February 2017 and October 2019 were included in the present study. Three patients presented with cSCC, whereas nine had OSCC. Archived formalin-fixed paraffin-embedded (FFPE) tissues from these 12 subjects were obtained from the Department of Oral and Maxillofacial Surgery of the Carol Davila Military Emergency Hospital in Bucharest, Romania. All participants provided written informed consent to take part in this study. Ethical approval for this retrospective analysis was obtained from the Ethics Committee of the Carol Davila Central Military Emergency Hospital. The study including all procedures and handling of patients' samples was carried out in accordance with the guidelines of the Declaration of Helsinki and national as well as institutional guidelines for research ethics. The clinical and histopathological characteristics of the SCC tumors were analyzed following the latest version of the international standard American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) system for the classification and staging of tumors. Presence of the EGFR T790M mutation was analyzed with the gb ONCO EGFR (T790M) Real-Time PCR assay (PN: 3245-048, Generi Biotech, Czech Republic); additional mutations in the EGFR-RAS-RAF mutations were tested using the droplet digital PCR (ddPCR) Screening assays for BRAF V600 (PN: 12001037), NRAS G12/G13 (PN: 12001627), NRAS Q61 (PN: 12001006), KRAS Q61 (PN: 12001626) mutations and EGFR exon 19 deletions (PN: 12002392) (all BioRad, Hercules, USA); details of these techniques are outlined below.

DNA extraction

The total genomic DNA was extracted from FFPE tissues (3 x 15-µM-thick sections) using the ReliaPrep™ FFPE gDNA Miniprep kit (PN: A2352, Promega). The protocol includes a proprietary deparaffinization method that does not rely on the use of xylene or any other hazardous solvents, ensuring at the same time the extraction of high-purity nucleic acids from FFPE samples. Following the deparaffinization of the FFPE tissue sections, the extraction protocol involves the lysis of the tissues. Nucleic acids released from the cells are further bound on the surface of a spin filter membrane and eluted in 50 µL of elution buffer after several rounds of washing. The purified genomic DNA was kept on ice and quantified using a Nanodrop spectrophotometer.

Droplet digital PCR

Before carrying out the ddPCR reaction, the genomic DNA samples were subjected to uracil-DNA-glycosylase treatment (UDG; PN: 1B1634, VWR) following the manufacturer's instructions. This step aimed to eliminate potential C>T transitions that could arise from tissue fixation, therefore reducing the risk of false-positive ddPCR reactions. All gDNA samples were analyzed in triplicate.

The ddPCR mixture requires the addition of 10 µL of 2x ddPCR Supermix for Probes (no dUTP) per reaction. The 2x ddPCR Supermix is a proprietary reagent containing an optimized reaction mix for primers and probes. Additionally, 1 µL of 20x BRAF (NRAS/KRAS/EGFR) primer and 3 µL of UDG-treated gDNA (7 ng/µL) were included in each ddPCR reaction, and the final reaction volume was adjusted to 20 µL by adding an appropriate volume of RNase/DNase-free water. Each analytical run included negative (wild-type control, WT) and positive controls at the same concentration as the analyzed gDNA samples. The positive controls were provided by Horizon Discovery (PN: HD701) and contained recurrent mutations in BRAF, KRAS, NRAS, cKIT, EGFR, and PIK3CA genes, at allelic frequencies ranging from 1% to 24.5%. The negative controls were WT genomic DNA samples acquired from Promega

Corporation (PN: G1471).

In the droplet generation step, individual reaction mixtures were loaded into disposable DG8 cartridges (Bio-Rad) along with 70 µL of droplet generation oil and passed through the microfluidic channels of a droplet generator. The resulting droplets from each well were transferred into a 96-well PCR plate, heat-sealed, and subjected to the following PCR conditions: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 98°C for 10 min. Post-PCR, the droplets from each well were analyzed using a QX200 droplet reader (Bio-Rad) and quantified based on target DNA fluorescence. The obtained data were analyzed using the QuantaSoftTM Analysis Pro software, version 1.0.596 (Bio-Rad). Additional details were reviewed recently [37].

Real-Time PCR

The EGFR T790M mutational status was evaluated using the CE-IVD validated gb ONCO EGFR (T790M) Real-Time (RT-) PCR kit (PN: 3245-048, Generi Biotech, Czech Republic). All reagents included in the kit were prepared for use according to the manufacturer's instructions. For each sample, we set two separate RT-PCR reactions. One reaction contained the Assay qPCR EGFR Control T790M and the other, the Assay qPCR EGFR MUT T790M, both in a total volume of 20 µL. 5 µL of the same gDNA sample (diluted to a final concentration of 50 ng/µL) was added to each of the two reactions. Thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 50 cycles with denaturation at 95°C for 10 s, and annealing/elongation at 60°C for 20 s. Fluorescence acquisition was set on the FAM/SYBR channel. All gDNA samples were analyzed in triplicate for the detection of EGFR T790M mutation. Each run contained two types of positive controls according to manufacturer instructions. We included one EGFR wild-type (WT) control and one EGFR T790M positive control, with 5 µL of the provided kit standards pipetted to each. Finally, to check the purity and quality of the PCR reagents, we introduced a third assay control, the so-called 'negative control'. In this case, RNase/DNase-free water was used to replace the corresponding volume of gDNA. The qRT-PCR assays were run on the Rotor-Gene Q 5plex HRM equipment (Qiagen, Germany) and the results were analyzed using the Rotor-Gene Q Series Software (version 2.3.1) (Qiagen, Germany). For each sample, the assessment of the mutational status was based on calculating fold change (FC) values from the Ct values obtained in the two separate reactions, as per the manufacturer's specifications.

Table 1: Demographic and clinicopathological data of SCC patients

Patient #	Gender	Age (years)	Area	Tobacco usage	Alcohol use	The primary site of the tumor	HPV infection	TNM	T stage	Lymph node metastases	Outcome
1	M	80	rural	no	no	lower lip	no	I	1	0	alive
2	M	67	urban	yes	no	tongue	no	IV A	3	2	dead due to disease progression
3	M	50	urban	yes	yes	tongue	no	IV A	2	3	alive
4	M	65	rural	no	yes	lower lip	no	I	1	0	alive
5	M	45	urban	yes	no	lower lip	no	III	3	1	alive
6	F	54	urban	yes	no	tongue	no	II	2	0	alive
7	M	60	rural	no	no	retromolar trigone	no	IV A	4	5	dead due to disease progression
8	M	69	urban	yes	yes	buccal mucosa	no	IV A	3	1	alive
9	F	85	rural	no	no	lower lip	no	II	2	0	alive
10	M	88	urban	no	no	cSCC of the parotid region	no	III	3	1	alive
11	M	51	rural	yes	yes	cSCC of the nasal region	no	IV A	4	2	dead due to disease progression
12	F	86	urban	no	no	cSCC of the genian region	no	I	1	0	alive

The limit of detection (LoD) declared for this assay is 5 copies per 25 µL reaction. This corresponds to a minimum detectable mutant allelic fraction of 0.05% in a background of 100,000 WT copies.

Statistical analysis

Data analysis was performed using The GraphPad Prism software (Boston, Massachusetts USA, www.graphpad.com) version 10.0.0. Patients' demographic and clinicopathological data were analyzed by descriptive statistics.

RESULTS

Patients' general characteristics

Table 1 describes the demographic and clinicopathological characteristics of the SCC patients. The clinical cohort consisted of 12 patients diagnosed with SCC of the head and neck region, encompassing 9 men (75%) and 3 women (25%). Among them, nine patients presented with OSCC (Patients #1-#9), while three subjects had cSCC (Patients #10-#12). The median age of the patients was 66 years, ranging from 45 to 88 years.

Fifty-nine percent (n=7) of the analyzed patients are from an urban environment, and 50% (n=6) of them are active smokers. Additionally, 33% (n=4) of the patients declared that they frequently consume alcohol, and 58% (n=7) presented with pathologically confirmed lymph node metastasis. Five patients were diagnosed with TNM stage IVA disease, three with stage I, and two patients each with stages II and III. All patients tested negative for the presence of high-risk HPV strains.

DNA extraction

The average concentration of DNA extracted from the archived FFPE tissues was 255.7 ng/µL (ranging from 70.2 to 484.3 ng/µL), and the average A260/A280 value was 1.83.

The A260/A280 values obtained for gDNA from FFPE tissues are within the optimal range (~1.8), indicating that the extracted gDNA is free from protein contamination. In addition, all A260/A230 values fall within the recommended range (2.0-2.2), indicating that gDNA has minimal contamination by polysaccharides. These observations confirm the obtained gDNA's suitability for subsequent molecular manipulations.

ddPCR results

The ddPCR analysis demonstrated that BRAF V600, KRAS Q61, NRAS G12/G13, and NRAS Q61 mutations were not present in the examined samples, emphasizing the rarity of these genetic alterations in the pathogenesis of squamous cell cancers. Figure 1 illustrates the absolute quantification of the NRAS Q61 mutation in FFPE samples using the ddPCR system. In addition, by exploiting the ultrahigh sensitivity of ddPCR technology, we successfully identified the presence of the EGFR Ex19Del mutation in a cSCC tumor developing in an 88-year-old male diagnosed with stage III disease (Figure 2). None of the remaining 11 SCC patients within the clinical cohort exhibited this particular mutation.

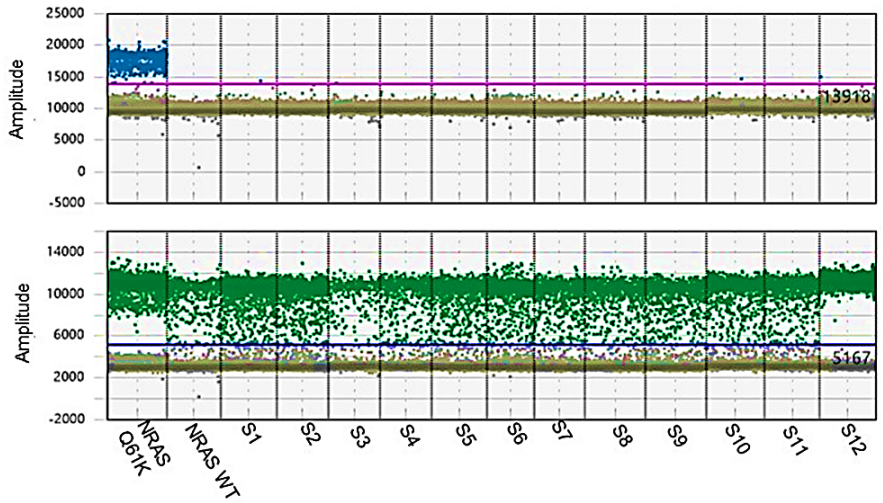

Figure 1: One-dimensional (1D) droplet fluorescence plot for NRAS Q61 mutation analysis. Blue dots represent the FAM-labeled droplets containing the NRAS Q61 -mutant allele, while green dots depict the HEX-labeled fluorescence probes associated with the NRAS WT allele. The pink line represents the threshold between positive and negative signals of the droplets. This determination enables precise NRAS Q61 mutation quantification in FFPE samples (S1-S12- SCC samples; NRAS Q61K- positive control; NRAS WT- negative control).

Real-Time PCR results

The 12 SCC samples were additionally subjected to qRT-PCR analysis to detect the EGFR T790M mutation. All SCC samples included in the study tested negative for the presence of the EGFR T790M mutation.

According to the manufacturer's recommendations, each analytical run included a positive control (containing the investigated mutation at an allelic frequency of 1%), a WT control (where only the WT allele was amplified), and a negative control (without gDNA), to investigate the purity and the quality of the reagents used. Figure 3 illustrates the

positive control of the EGFR T790M RT-PCR assay, the negative control along with a sample negative for this mutation (EGFR wild-type; in blue).

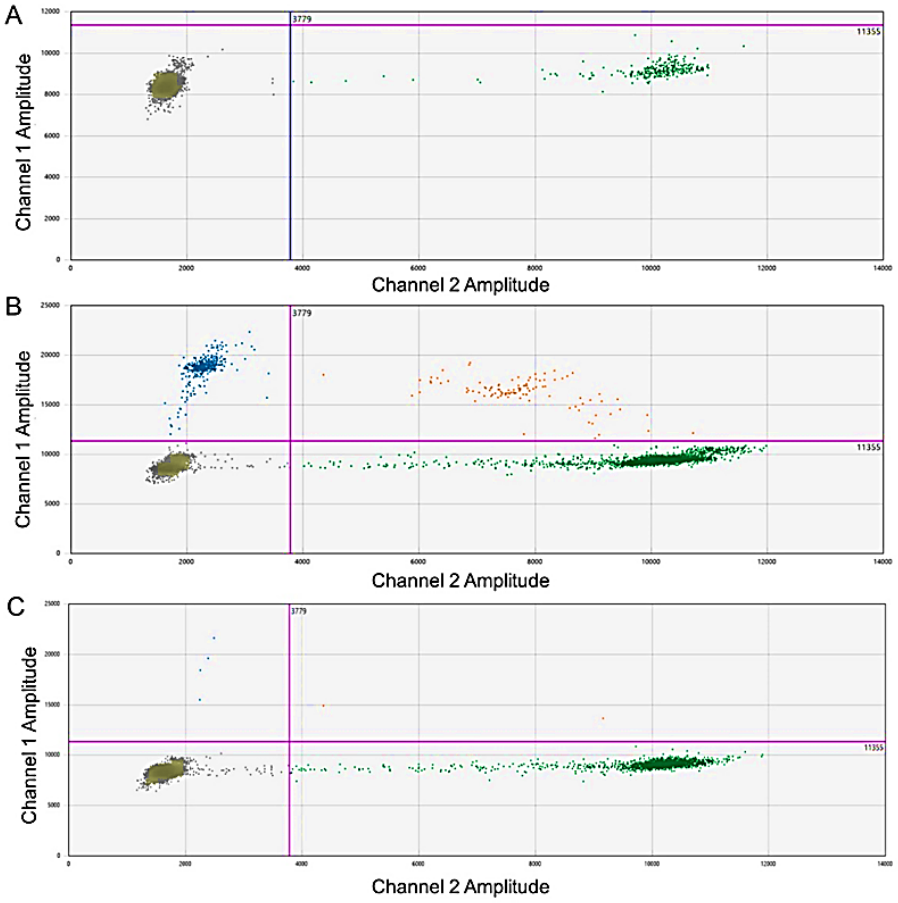

Figure 2: Two-dimensional (2D) ddPCR plots of EGFR Ex19Del mutation detection in FFPE samples. (A) ddPCR results for an EGFR wild-type DNA sample (negative control). The 2D plot illustrates that no droplets are positive for EGFR Ex19Del mutant alleles on channel 1 (FAM labeled, in blue) while EGFR Ex19 wild-type alleles are abundantly detected on channel 2 (HEX labeled, in green). Empty droplets (in grey) do not contain the EGFR Ex19Del target amplicon. (B) ddPCR results for the positive control (presenting the EGFR Ex19Del mutation). Mutant alleles are detectable on channel 1 (FAM labeled, blue). Double-positive droplets (orange) contain both mutant and wild-type EGFR Ex19 alleles. (C) ddPCR results for the cSCC tumor tested positive for EGFR Ex19Del.

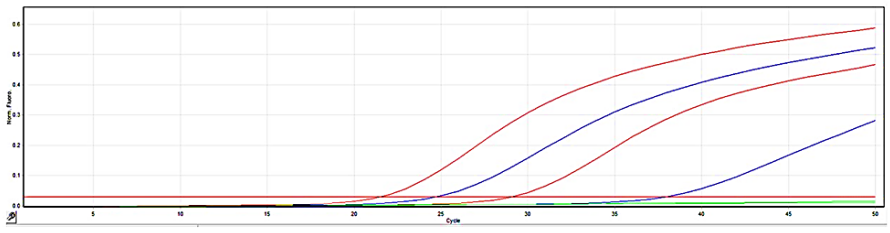

Figure 3: Determination of the EGFR T790M mutational status by RT-PCR. The positive control of the qRT-PCR analysis is marked in red, whereas the negative control is in green. The amplification curve of the EGFR WT SCC sample is depicted in blue. The threshold for the entire run was set at 0.03. A sample can be considered mutated when its FC value is lower than the cut-off FC value and the difference between the CtM of a sample and the CtC of the WT standard is lower than 13. If the latter condition is not met, or the FC value of a sample is higher than the cut-off FC, the sample is evaluated as WT.

DISCUSSION

SCC neoplasms of the head and neck are one of the most heterogeneous and aggressive tumors ever described [10]. Despite recent advances in SCC diagnosis and therapy, the prognosis of advanced patients remains poor, highlighting the need for a more comprehensive evaluation of these tumors [38–40]. From the beginning of modern medicine until now, SCCs have always been approached separately, depending on the tissue from which they originate (e.g., cutaneous SCC, oral SCC) or the anatomical region where they occur. However, in the present study, we wanted to deliver a unifying approach regarding cSCCs and HNSCCs, by analyzing the shared molecular mechanisms involved in their pathogenesis. Our point of view is also supported by the fact that the cSCC and HNSCC pathogenesis converge toward the same risk factors and genetic alterations [2,41]. Additionally, we believe this approach may facilitate a comparative examination of intrinsic tumor cell heterogeneity, stromal composition, and therapeutic efficacy across SCCs occurring in different anatomical sites, an idea that is attracting considerable research interest from the international community as well [2].

Here, we embarked on a preliminary analysis of the frequency and role of hotspot mutations in the EGFR-RAS-RAF signaling components within a clinical cohort encompassing 12 Romanian patients diagnosed with SCCs (3 cSCCs and 9 OSCC cases). We employed the third-generation PCR technology, also known as ddPCR, to screen for mutations such as BRAF V600, NRAS G12/G13, NRAS Q61K, KRAS Q61, and EGFR Ex19Del, while the mutational status of EGFR T790M was assessed using RT-PCR. None of the examined tumor specimens exhibited mutations in the BRAF, KRAS, and NRAS genes. Our findings are consistent with previous observations in the field, supporting the assertion that these genes are rarely mutated in cSCCs and HNSCCs [34,42].

The analysis of the demographic data of the subjects enrolled in the study revealed that 75% of them are men (n=9) and half (n=6) are smokers. We also observed that 58% of the subjects (n-=7) have metastases with lymph node involvement and all SCC tumors are HPV-negative (n=12).

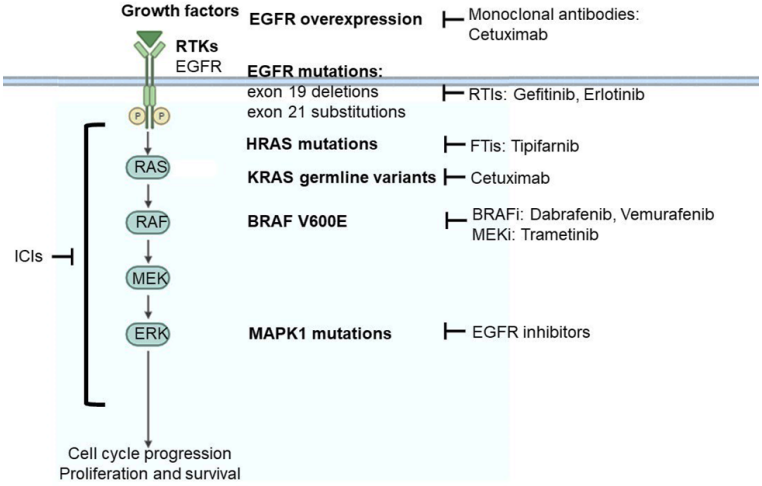

Figure 4: Precision treatment strategies for EGFR/MAPK pathway-mutated head and neck squamous cell neoplasms. ICIs- Immune checkpoint inhibitors; RTKs- Receptor tyrosine kinases; RTIs- Receptor tyrosine kinase inhibitors; FTIs- Farnesyl transferase inhibitors; BRAFi- BRAF inhibitors; MEKi- MEK inhibitors

However, among the 12 FFPE samples analyzed for EGFR Ex19Del, one HPV-negative cSCC tumor identified in the parotid region of a male tested positive for this mutation. Interestingly, the patient presenting with this tumor is an 88-year-old man from an urban environment who neither smokes nor drinks alcohol. Nevertheless, current literature emphasizes the role of HPV infection status as a valuable prognostic indicator for HNSCC/cSCC patients. Usually, HPV-negative SCCs display alterations in the tumor suppressors such as p53 and cell cycle regulators and present with an unfavorable prognosis compared to HPV-positive tumors, which show less genomic complexity [43]. Several research groups have examined the prevalence of EGFR Ex19Del mutations in head and neck SCCs, reporting a 1% occurrence in the Caucasian population [44] and 7.3% in the Asian population [45]. Despite providing tumor cells with different adaptive advantages via downstream hyperactivated pathways (ERK/MAPK, PI3K/Akt, and JAK/STAT), these mutations also confer sensitivity to TKIs [3]. Several ongoing clinical trials are investigating TKIs' clinical safety and efficacy in cSCC/HNSCC. Single-target TKIs gefitinib and erlotinib given as monotherapy have not demonstrated clinical efficacy in

HNSCC and cSCCs [46,47]. However, remarkable benefits have been reported when small-molecule TKIs were administered in conjunction with other therapies such as cisplatin, docetaxel, or immunotherapy [31,48]. Therefore, these mutations might be exploited for targeted pharmacological interventions in the future, once the most effective drug combinations are identified.

The evaluation of the mutational status of the MAPK signaling pathway is another hot topic in the field of SCC bio-medical research. Several recent studies potentiated that mutations in different components of the MAPK signaling pathway, such as BRAF and RAS, may serve not only as important therapeutic targets but also as valuable indicators of prognosis in SCC, indicating those patients who are most likely to respond to immunotherapy (Figure 4) [36].

This is why we focused our research on investigating these mutations in a small group of patients diagnosed with SCC of the skin and oral mucosa, the present study being also the first one of this type conducted in Romania. Recent studies have shown that even in p53-mutated HNSCC tumors, notorious for their unpredictable evolution, MAPK pathway mutations may help identify those patients with superior outcomes [36]. It is also worth mentioning that the positive prognostic value of these mutations in HNSCC has been reported independently of HPV status [36].

Bioinformatic analysis of HNSCC patient data retrieved from The Cancer Genome Atlas (TCGA) revealed that MAPK-mutant HNSCC tumors present with an increased infiltration of CD8+ T cells and dendritic cells and immune-active cytolytic and interferon-gamma signatures in situ, data that were also reported by our group [36,38,49].

Those features are not observable in HNSCC tumors with mutations in the PI3K, WNT, NOTCH, JAK/STAT, NF-kB, or TGF-beta signaling pathways [36]. Moreover, independent of tumor mutational burden (TMB), MAPK pathway mutations alone predicted those HNSCC patients who benefited the most from clinical anti-PD1/PD-L1 immunotherapy [36].

Similar prognostic roles of MAPK pathway mutations were reported in pan-cancer immunotherapy studies, suggesting that the mutational analysis of this signaling pathway might be essential to improve SCC patient risk stratification.

CONCLUSIONS

Oral and cutaneous squamous cell neoplasms (SCCs) remain a public health challenge and a topic of current scientific interest. Genetic analysis is essential to understand the biology and the clinical behavior of SCCs, unraveling potential biomarkers and therapeutic targets with practical applications in this challenging disease. Therefore, to align with the precision medicine initiatives in the field, we initiated a clinical study to explore the prevalence and significance of hotspot mutations in the EGFR-RAS-RAF signaling pathway within a small clinical cohort of Romanian patients diagnosed with squamous cell neoplasms of the head and neck. Taking into account the shortcomings reported with traditional chemotherapies, administered on a 'one size fits all' basis and resulting in poor efficacy, limited selectivity, and increased toxicity in HNSCCs/cSCCs, we are actively supporting the transition towards the adoption of more individualized anticancer agents in SCC clinical practice.

The present retrospective study emphasizes the absence of mutations in the NRAS, BRAF, and KRAS genes within a small clinical cohort of cSCC and HNSCC Romanian patients, marking the first study of this type conducted in Romania. In addition, our research underlines the remarkable sensitivity of the third-generation PCR technology, the so-called 'droplet digital PCR' (ddPCR), which may be instrumental for detecting low-frequency actionable mutations in cSCC/OSCC tissue biopsies.

Conflicts of interest and sources of funding

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Authors' contribution

Conceptualization, M.N., and EG.D.; methodology, EG.D.; software, A.C, and C.Constantin.; validation, A.N.M., C.Constantin., M.S. and C.S.; formal analysis, C.Caruntu.; investigation, A.C.; resources, M.N. and C.Carruntu.; data curation, EG.D.; writing—original draft preparation, EG.D., A.C., A.N.M., M.S., C.Constantin, C.Caruntu, C.S., M.N.; writing—review and editing, M.N. and C.Caruntu.; funding acquisition, M.N., and C.Caruntu. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee from Carol Davila Central Military Emergency Hospital, Bucharest (No. 333/28.03.2019).

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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