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Original scientific paper

PATHOGENIC SOMATIC ALTERATIONS IN ADVANCED HPV-NEGATIVE CELL SQUAMOUS LARYNGEAL CARCINOMA REVEALED VIA TARGETED NEXT GENERATION SEQUENCING

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During the last decade, next generation sequencing (NGS) became the major tool for detection of somatic mutations in cancer, but data about mutations in advanced laryngeal squamous carcinomas is still scarce. The aim of this study is to analyze the mutation profile of key oncogenes and tumour suppressor genes in advanced HPV-negative laryngeal squamous cell carcinoma. A total of 57 Bulgarian LSCC patients were included. DNA was isolated from fresh-frozen tissues. Targeted NGS was performed using TruSeq Amplicon Panel on Illumina platform, and data was analysed with VarSeq Software. Results revealed altogether 92 known pathogenic and likely pathogenic variants in 27 tumour-associated genes. Thirteen new variants were predicted to be pathogenic with four or more prediction programs. The most frequently mutated gene was TP53, with mutations in 84.2%, followed by MET in 19.3%, CDKN2A in 15.8%, PIK3CA in 14% and FBXW7 in 8.8% patients. Interestingly in eight of the supraglottic LSCC patients we found two TP53 mutations and in one subglottic LSCC patient – three TP53 mutations. For the first time the mutational spectrum of three LSCC sub-locations was analyzed and the supraglottis LSCC showed

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more mutated genes (n=20) compared to glottis (n=10) and subglottis (n=12) tumours. In 9% of the samples we found combined high-risk TP53 and RAS mutations, previously associated with poor overall survival and drug resistance. The analysis revealed that NOTCH1 mutations are not common for LSCC in comparison to other HNC loci. In addition, we found three common polymorphisms in TP53 (p.Pro72Arg), KDR (p.Gln472His) and KIT (p.Met541Leu) genes, strongly associated with angiogenesis, poor prognosis and metastasis and drug sensitivity in cancer diseases. In conclusion, NGS targeted sequencing enables discovery of new mutations in key genes relevant to LSCC, some of which might be useful in selection or development of more precise LSCC treatment approaches in the future.

Keywords: Advanced laryngeal cancer, HPV-negative, NGS targeted sequencing, TP53, somatic mutations

INTRODUCTION

Next Generation Sequencing (NGS) is the major tool for detection of genome and genetic variations in tumours, research and diagnostics of cancer diseases (GRUMBT *et al*., 2013). Somatic mutation screening with NGS of head and neck cancers (HNC), the 6th most common cancers in the world, was performed in several studies (MORRIS *et al.,* 2017; NICHOLS *et al*., 2012; STRANSKY *et al.,* 2011; AGRAWAL *et al.,* 2011), but the precise mechanism of laryngeal cancerogenesis is still elusive. Differences in mutation mechanisms between HNC tumour locations have been presented previously on genomic, epigenomic and genetic level. SHIGA *et al.* (2012) reported that LSCC (laryngeal squamous cell carcinoma) showed significantly higher frequency of LOH and lower p16 gene promotor methylation status in comparison to oral squamous cell carcinoma (OSCC) (SHIGA *et al.,* 2012). In the study of PERI *et al*. (2017) was reported that TP53 mutations were predominant in oral and laryngeal cancer (PERI *et al*., 2017), whereas CASP8 gene has significantly less mutated in LSCC (CHAU *et al.,* 2016) comparison to other HNC locations. In addition, mutations in NSD1 gene were concentrated in laryngeal cancers and the association of NSD1 mutation with better overall survival was specific to laryngeal tumors (PERI *et al*., 2017) unlike other HNC locations. These findings suggest a biological difference between the HNC subsite tumours. Laryngeal cancer may have different mechanisms of cancerogenesis in comparison to the rest HNC tumours, needing detailed investigation.

Laryngeal cancer, the second most common HNC, in 95%-98% is from epithelial origin, has not shown overall survival improvement in the last decades (MÄKITIE and MONNI, 2009). In 2012, 156 887 cases and 83 376 deaths were registered worldwide (GLOBCAN, 2012 data for both genders). Well known cancer risk factors are the abuse of tobacco and alcohol concentrate products. Human papilloma virus (HPV) was established as an additional risk factor for the development of HNC, mainly for oropharyngeal cancers, and is associated with improved overall and disease-specific survival (ANG *et al*., 2010). In laryngeal cancer, the role of HPV is not so evident. CHEN *et al*. (2017) found HPV in 13.2% of 109 laryngeal tumour specimens (CHEN *et al.,* 2017), whereas in another recent study in laryngeal cohort comprised of 82 LSCC patients, no HPV was found (ONERCI CELEBI *et al*., 2018). However, the majority of NGS studies explored LSCC in the general HNC group, and only few of them investigated LSCC as a

separate group (MANTEROLA *et al.*, 2018; FANJUL-FERNÁNDEZ *et al*., 2013), which may hinder the elucidation of specific molecular characteristics of LSCC.

The genetic landscape of HNC observed so far is heterogenious, with mutations in various genes, differing dependent on the pathways engaged and the involvement of HPV in the pathogenesis. For example, overall HPV-negative and positive HNC cluster into two different subgroups with few overlapping mutations: HPV-negative exhibit mutations in TP53, CDKN2A, MLL2/3, NOTCH1, PIK3CA, NSD1, FBXW7, DDR2, and CUL3; HPV-positive exhibit mutations in PIK3CA, MLL3, DDX3X, FGFR2/3, NOTCH1, NF1, KRAS, and FBXW7, as seen in Seiwert *et al*. (2015). The best-studied gene for HNC as well as LSCC is TP53. Mutations in TP53 are related to HPV-negative tumours and associated with poor outcome and chemotherapy resistance (SEIWERT *et al.*, 2015). In order to establish new therapeutic molecular markers and improve treatment of LSCC, more detailed understanding of the molecular mechanisms involved in the cancerogenesis of this aggressive disease is needed.

The aim of the current study is to explore by targeted NGS the profiles of somatic mutations of HPV-negative advanced LSCC tumours and to peek into the three laryngeal locations (supraglottis, glottis and subglottis) in Bulgarian patients. The results could offer insights into the mechanisms of cancerogenesis and identify new potential biomarkers for diagnosis and prediction. They would also facilitate stratification and enrollment of LSCC patients in clinical trials of new targeted therapies, leading to better treatment.

MATERIAL AND METHODS

Patients and tissue samples

In the current study a total of 57 patients diagnosed with advanced LSCC were enrolled. Patients were recruited at the Otorhinolaryngology Department, University Hospital "Queen Joanna - ISUL" Sofia during the period 2013-2015. All participants signed written informed consent forms, and the protocols №: 432/ 2017 and №: 435/2017 of the current study was approved by the Ethics Committee of Medical University of Sofia. Tumour tissue samples from each patient were obtained during surgery and frozen in liquid nitrogen (-196ºC) within 15 minutes. Samples were transported and stored at -80ºC at the biobank of the Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical University of Sofia, Additional material was taken for immunohistochemistry tests to determine the pathomorphological characteristics of the samples. None of the patients in this study has received chemotherapy or radiotherapy before surgery.

HPV testing

Analyses were performed with digene HPV Genotyping RH test (Qiagen, Hilden, Germany) that included 2 kits for amplification and detection by hybridization of 18 high risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 и 82). The results were confirmed by nested PCR with MY/GP primers.

DNA extraction

DNA was isolated from LSCC tumour tissues with QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The quality and quantity of DNA

samples were evaluated with 0.8% agarose gel and NanoDrop ND 1000 (Thermo Fisher Scientific, Waltham, USA). After the quantification, working aliquots of 50ng/µl were prepared for the next steps. The DNA was measured on Qubit v 2.0 (ThermoFisher Scientific, Waltham, USA) (fluorometric method).

Targeted NGS: TruSeq Amplicon-Cancer Panel (TSACP)

Sequencing amplicon libraries were prepared using the TruSeq Amplicon Cancer Panel (Illumina, California, USA), according to the manufacturer's instructions. The panel generates 212 amplicons from 48 cancer-related genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, and VHL. This gene panel was made according to relevant publications, and late-phase pharmaceutical clinical trials and guidelines from the College of American Pathologists (CAP) and National Comprehensive Cancer Network (NCCN).

Preparation of libraries

DNA input of 250 ng from each sample was used for library generation. Sets containing pairs of oligonucleotides (oligonucleotide pool) specific to the targeted regions were briefly hybridized to each genomic DNA sample. Amplicons were generated by extension and ligation of bounded oligonucleotides using a DNA polymerase and ligase. This was followed by PCR amplification. The PCR primers were flanked by index sequences for sample multiplexing as well as common adapters for sequencing cluster generation. For library assessment agarose gel electrophoresis was performed and expected PCR product sizes were observed. Each sample library was cleaned up and normalized according to the manufacturer's instructions. Pooling of equal volumes of each library sample generated the final sequencing library.

Sequencing and bioinformatics analysis

The pooled library was sequenced on a MiSeq machine, Illumina using a 2 x 151 paired end sequencing design. MiSeq Reporter was used for generation of VCF files. Variant annotation to the human reference genome (GRCh-37/hg19), filtering and interpretation was performed using VarSeq software (Golden Helix, Montana, USA). Cut off of 5% for variant allele frequency (VAF) and read depth of 100 were used for filtering in order to exclude false positive variants. In the analysis different databases like ExAC, Cosmic, dbSNP, ClinVar, RefSeq and many others were used. Variant prediction programmes like SIFT, Polyphen2, Provean, Mutaiton Taster, Mutation Assesor, MetaSVM, FATHM, LRT, Vest3 were also used in order to predict pathogenicity of newly identified variants.

RESULTS

Clinicopathological characteristics of the patients

The mean age in the study group was 62 years (range, 41-84 years). The cohort comprised 3 female and 54 male patients, in total 57 patients, all of whom diagnosed with

advanced squamous cell carcinoma of the larynx. Distribution according to T classification was as follows: T3–20 patients (35%) and T4–37 patients (65%). Histologically verified lymph node metastases were present in 25 patients (44%) at the time of surgery. 26 LSCC patients were supraglottic (46%), 22 glottic (39%) and 9 subglottic (15%). Fifty patients report excessive use of tobacco and 39 report alcohol abuse.

HPV testing

The analysis was performed with a ready to use kit for detection of viral DNA. For validation of the results, we used nested-PCR with MY09/MY11 and GP5+/GP6+ primers, with a positive control. HPV testing for all of the patients was negative.

Mutational status in 57 advanced HPV-negative LSCC patients by TSCAP, Illumina

From the analysis of MiSeq, Illumina data report, we found altogether 92 pathogenic variants in 27 cancer associated genes (Supplement 1). The results show 13 unpublished variants, and 79 published mutations. The new variants are predicted as pathogenic by more than four prediction programs, and we consider them with a likely pathogenic effect.

In addition, we investigated 2 polymorphisms (one in TP53 and one in KDR genes), reported in COSMIC database as neutral One of the neutral variants is the well-known TP53 benign polymorphism rs1042522, c.215C>G (p.Pro72Arg), related to chemotherapy outcome (ZHENG *et al.*, 2014). In 60% of the cases (n=34) the benign missense variant p.Pro72Arg (rs1042522) was found in homozygous Arg/Arg state, whereas in 26% (n=15) the variant was present in heterozygous Pro/Arg state. In a smaller group of 14% (n=8) the variant was in Pro/Pro homozygous state. The other neutral variant, rs1870377 in the KDR gene, c.1416A>T (p.Gln472His) was observed in 31.58% of the patients (n=18). In six of the patients, rs1870377 was in homozygous TT genotype, and in 12 patients was found in heterozygous AG genotype.

A polymorphism–rs3822214 (c.1621A>C; p.Met541Leu) in exon 10 in the KIT gene was found. It is reported in ClinVar as likely benign, but as pathogenic in COSMIC database (COSM28026). It was evaluated as tolerated by five programs for in silico predictions. p.Met541Leu was observed in 16% of the LSCC patients (n=9), and six of them were positive for nodal metastasis at time of surgery.

All observed pathogenic variants include 59 missense variants, 12 frameshifts, 11 stop gains, 2 inframe deletions, 4 splice acceptor and 4 splice donor variants. Of all 57 investigated LSCC patients, 84.2% harbored mutations in TP53 (n=48). Intriguingly, in eight patients (A59, A63, A38, A9, 42, A30, A45), in parallel two different mutations in TP53 were found, and in one patient (A5) three different TP53 pathogenic variants were revealed. In nine patients TP53 variants with pathogenic prediction were not found (A2, A16, A17, A20T, A53, B2T, B10T, B12, B14T). The second most commonly mutated gene was MET–19.3% (n=11), followed by CDKN2A–15.8% (n=9), PIK3CA with 14.0% (n=8) and FBXW7 with 8.8% (n=5). Equal mutation frequency demonstrated ATM, GNAS, HRAS, PTEN, FGFR2 and KIT with 5.3% (n=3). Two patients had mutations in JAK3, KRAS, SMAD4, APC, BRAF and SMARCB1, and one mutation was found in CDH1, ERBB4, FLT3, GNAQ, KDR, NOTCH1, NRAS, RET, STK11 and VHL. In four patients we did not find any pathogenic variants (A20T; B2T; B10T;

B14T). Figure 2 shows the distribution and frequencies of the pathogenic variants detected in the studied LSCC group.

A total of 45 mutations and unpublished variants with pathogenic predictions in TP53 gene were found, the majority of which were missense (n=21). The TP53 spectrum also included ten stop gains and seven frameshifts, two of which new, c.239_247delinsA (p.Pro80>LeufsTer48) and c.250_265del (p.Ala84ProfsTer34). In addition, four splice donor variants were observed: one with a new nucleotide change (c.96+1G>A) described in dbSNP germline pathogenic variant rs1131691003 in hereditary cancer-predisposing syndrome; moreover, two known splice acceptor variants; and one nondescribed inframe deletion in exon 10: c.1041_1064del (p.Leu384_Ala355del).

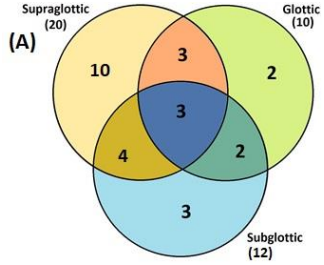
For the next frequently mutated gene–MET, we analyzed only two known variants, both of them reported as pathogenic in COSMIC database–c.3029C>T (p.Thr1010Ile) and c.1124A>G (p.Asn375Ser). The p.Thr1010Ile mutation, highly recurrent in our investigated group and was found in 10 (17.5%) LSCC patients.

In CDKN2A gene, the analysis revealed seven described previously pathogenic variants in nine samples, five of them located in exon 2. CDKN2A mutations include two missense, splice acceptor and frameshift variants and one intron variant. Five PIK3CA variants were found. Four of them are known missense pathogenic variants and one is a new frameshift c.3031delC (p.Pro1011fsTer16). Four missense and one stop gained variants in FBXW7 gene: c.1136A>G (p.His379Arg), c.1428C>G (p.Ser476Arg), c.1877C>T (p.Ala626Val), c.1513C>G (p.Arg505Gly) and c.1435C>T (p.Arg479Ter), were found. Moreover, new variants with pathogenic predictions were found also in CDH1, ERBB4, FLT3, GNAQ, KDR, NRAS and SMARCB1. Table 1 (Supplement 1) represents all variants with pathogenic prediction that we discovered.

Distribution of pathogenic somatic variants across supraglottic, glottic, and subglottic laryngeal cancers

For the first time we explore and describe in detail variants with pathogenic prediction across the three main laryngeal locations: supraglottis, glottis and subglottis. In our study we investigated 27 supraglottic, 21 glottic and 9 subglottic LSCC samples. As mentioned previously, in three supraglottic and one glottic LSCC patient we did not find any variant with pathogenic prediction.

The supraglottic LSCC was the most often mutated location with variants detected in 20 genes, whereas in the glottic and subglottic LSCC groups pathogenic variants in 10 and 13 genes were found, respectively. We summarize results in Venn Diagram as published previously (HEBERLE *et al*., 2015). The Figure 1 shows the number of non-overlapping and overlapping genes across LSCC locations and table with detailed presentation of affected genes in each location separately and in groups.



(B)

LSCC Locations	Affected Genes
Supraglottic	FBXW7; HRAS; FGFR2; CDH1; ERBB4; FLT3; GNAQ; KDR; NOTCH1; NRAS
Glottic	RET; STK11
Subglottic	KIT; PTEN; VHL
Supraglottic & Glottic	CDKN2A; PIK3CA; SMARCB1
Supraglottic & Subglottic	JAK3; KRAS; SMAD4; BRAF
Subglottic & Glottic	ATM; APC
All Locations	TP53; MET; GNAS

Figure 1. (A)Venn Diagram presenting the number of overlapping and unique mutated genes; (B) Table with detailed list of affected genes across the three LSCC locations

The variants in TP53, MET and GNAS were similarly distributed among the three LSCC locations. Interestingly, all of the patients with two parallel pathogenic variants in TP53 were with supraglottic location and the patient with three TP53 variants was diagnosed with subglottic cancer. Although, CDKN2A and PIK3CA variants were detected in both supraglottic and glottic LSCC location, the patients with supraglottic prevailed, in seven and five patients, respectively. All FBXW7 variants were in supraglottic LSCC, whereas ATM variants was not found in the same location. Mutated HRAS was observed in two supraglottic and one subglottic LSCC location. All KIT, PTEN and a VHL gene variants were found only in subglottic LSCC, while all three FGFR2 and one pathogenic variant in each of CDH1, ERBB4, FLT3, GNAQ, KDR, NOTCH1, and NRAS were observed in supraglottic LSCC. None of the BRAF, JAK3 and KRAS variants were found in glottic LSCC, and APC variant was not detected in supraglottic LSCC. Mutated RET and STK11 were in glottic LSCC, and one SMARCB1 variant was found in supraglottic and glottic LSCC. Figure 2 shows the frequency and distribution of the observed variants across all laryngeal locations in the studied advanced LSCC group. Most of the analysed mutated genes are presented with few variants, but the results could give us idea about the affected genes in each LSCC locations.

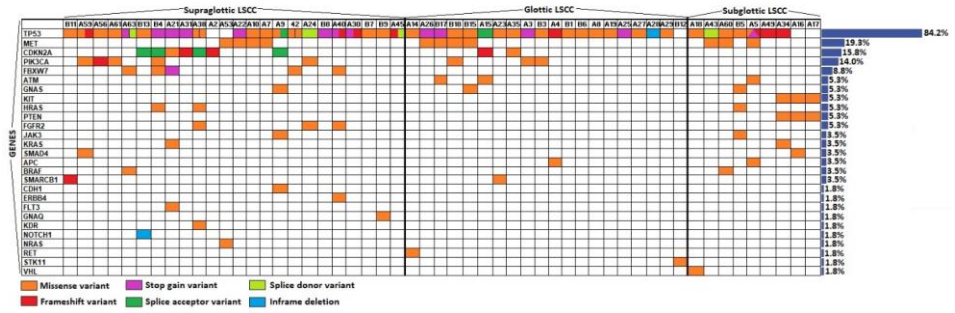


Figure 2. Distribution and frequency of the explored variants in 57 HPV-negative LSCC samples, divided by tumour locations. Tumour samples are in columns, genes are in rows.

DISCUSSION

The aim of our study was to explore the mutational status of advanced HPV-negative tumour samples from 57 LSCC patients with targeted NGS, (TruSeq Amplicon-Cancer Panel) on MiSeq, Illumina platform. In addition, for the first time we report comparison of the genetic changes between the three main laryngeal locations: supraglottis, glottis and subglottis. Next to the presented pathogenic variants, we highlight other three variants in TP53, KDR and KIT genes not considered as pathogenic in databases, but related with cancerogenesis and drug therapy.

The only targeted therapy drug for modelling of HNC is cetuximab, approved by FDA (Food and Drug Administration, USA) in 2006. It is IgG1 monoclonal antibody inhibiting the ligand binding of EGFR (Endothelial Grow Factor Receptor), which is overexpressed up to 90% of HNC patients (AGULNIK, 2012). However, cetuximab is not suitable drug for each HNC patients and is associated with higher CSM (Cancer Specific Mortality) in comparison to other chemotherapy drugs (XIANG *et al*., 2018), resulting in more frequent interruption of treatment and high grade toxicity (BONNER *et al.*, 2006). Development of personalized targeted therapy will be beneficial for successful therapy of LSCC and HNC sites.

The most frequently mutated genes are discussed as well as their impact on laryngeal cancerogenesis and potential for treatment selection. The rest explored genes are found in only few patients and could give us the spectrum of affected genes.

Our results showed pathogenic variants in 27 cancer related genes, and the most frequently mutated gene was TP53 similar to other NGS head and neck cancer studies (NICHOLS *et al*, 2012; STRANSKY *et al.*, 2011; AGRAWAL *et al.*, 2011; SHIGA *et al*., 2012). Next commonly mutated were MET, CDKN2A, PIK3CA and FBXW7. The rest of the 22 mutated genes in our study were APC, ATM, BRAF, CDH1, ERBB4, FGFR2, FLT3, GNAQ, GNAS, HRAS, JAK3, KDR, KIT, KRAS, NOTCH1, NRAS, PTEN, RET, SMAD4, SMARCB1, STK11 and VHL. From one side the observed mutations confirm previous finding that LSCC is a highly heterogeneous disease (DE MIGUEL-LUKEN *et al*., 2016). On the other side, we found differences in the distribution and frequency of mutations between HNC locations, which may suppose internally heterogeneity of laryngeal loci. In comparison to oral squamous cell carcinoma (OSCC), where the mutational rate of TP53, MET and FBXW7 genes is 68%, 4% and 2% (ER *et al.*, 2015) respectively in our explored LSCC samples the three genes are presented with higher number of mutations: 84.2%, 19.3% and 8.8%. In addition, another NGS investigation on HNC by CHAU *et al.* (2016) published that NOTCH1 is the second most mutated gene after TP53 in p16-negative patients (CHAU *et al.*, 2016), whereas in our investigated LSCC group NOTCH1 mutation is found in only one patient. In the same study of CHAU *et al.* (2016), authors investigated significant number of HNC samples (n=213), but the enrolled LSCC samples were just 24. After detailed investigation of the article, we identified that among the analyzed 24 LSCC samples, NOTCH1 mutation was found in one patients, similarly to our results. Taken together these findings may suggest that NOTCH1 mutations are not common for laryngeal, but for oral and oropharyngeal sites, where the mutations appeared more frequently (CHAU *et al.*, 2016). Hence, laryngeal cancer may have distinct pathways and features of cancerogenesis from other HNC sites. Incorporation of NGS analysis in treatment management based on

characterization of LSCC genetic profiles, similar to other solid cancers (NAGAHASHI *et al*., 2019), is needed.

TP53 is one of the most mutated genes in solid cancer, but its involvement in cancer diagnosis and prognosis is still rare due to the controversial data of the effect of these aberations in cancer diseases (BRADFORD *et al.,* 1997). In HNSCC, TP53 mutations have been associated with negative HPV status and poor outcome after standard treatment (CABELGUENNE *et al*., 2000). Recently, NESKEY *et al*. (2016), developed a computational approach and score evaluation called Evolutionaty Action (EA) in order to determine the power of missense TP53 mutations as high- or low-risk. Moreover, the same research team demonstrated that in HNSCC patients the high-risk TP53 mutations are associated with aggressive cancer phenotype and decreased sensitivity to platina-based therapy, in comparison to those with wtTP53 or low-risk TP53 mutations (NESKEY *et al*., 2015). With the use of database http://mammoth.bcm.tmc.edu/cgi-bin/panos/EAp53.cgi (NESKEY *et al.,* 2015) we calculated prediction scores of the identified 21 missense LSCC TP53 pathogenic variants. Five missense variants (p.Met133Ile; p.Arg156Pro; p.His214Arg; p.Tyr236Asn and p.Arg282Trp) were determined to be low-risk TP53 mutations, and the remaining sixteen missense variants were predicted to be high-risk variants (p.Arg158His; p.His179Tyr; p.His179Arg; p.His193Arg; p.Leu194Arg p.Arg213Leu; p.Cys238Arg; p.Gly244Asp; p.Gly245Val; p.Gly245Cys; p.Arg248Leu; p.Arg248Gln; p.Arg248Trp; p.Arg273Leu; p.Cys275Phe and p.Pro278Leu). In addition, in patients with colorectal liver metastatic cancer carrying high-risk TP53 mutations in combination with mutation in RAS gene family were associated with shorter 5-year survival after surgery (CHUN *et al*., 2019). In our study, five patients (9%) carry mutations in HRAS or KRAS in combination with high-risk TP53 variants. Low- or high-risk TP53 mutations in addition to RAS mutations could be used as predictive and prognostic biomarker for treatment opportunities in HNC.

The next most frequently mutated gene in our study is MET. We identified two known MET mutations in 19.3% (n=11) of the cases, in SEMA and JM domains, c.1124A>G (p.Asn375Ser) and c.3029C>T (p.Thr1010Ile), respectively. In our study, the pathogenic variant p.Thr1010Ile was found in 17.54% of the investigated LSCC patients and previously has been shown as an activating "driver mutation" (MA *et al.,* 2008), while p.Asn375Ser is related with familial cancer history (TODE *et al*., 2017). Both of them are associated with deregulated MET signaling pathway. In our study, ten of the MET mutated patients reported tobacco smoking for more than twenty years, which may suggest relation between nicotine action and arising of MET mutations. Additionally, according to TU *et al*. (2018), patients with an activated MET gene, which are also positive tobacco smokers, are more sensitive to MET inhibitors (TU *et al*., 2018). In HNC cell lines, inhibition of MET with SU11274 can synergize showing a great efficacy with erlotinib and cisplatin chemotherapy, and can be significantly superior compared to the agent alone (SEIWERT *et al*., 2009). Analysis of MET gene status could be suitable for selection of more precise cancer treatment options.

In HNC, CDKN2A is known to be altered through different mechanisms, including absence of protein expression, homozygous deletion, and promoter hypermethylation (LIM *et al.*, 2014). The clinical impact of the various potential mechanisms of CDKN2A inactivation in LSCC is not fully revealed. In a previous study of our research team the mutational status of TP53 and CDKN2A was examined in a large LSCC cohort (n=108), and in 16 patients (14.2%)

genetic aberrations in CDKN2A were detected (TODOROVA *et al*., 2015). In the present study, we further support this finding as we identified similar percentage (15.8%) of the patients carrying pathogenic variants in CDKN2A. Mutations in CDKN2A are significantly associated with high p16INK4a expression in HPV-negative LSCC (LOO *et al.*, 2003), and are a predictor for good response to immunotherapies (HELGADOTTIR *et al.*, 2018). We found that most of the mutations appear in exon 2, which affect both p16 and p14/ARF genes, which have a critical role in cell cycle progression.

Analysis of WES data in HNC showed that the PI3K pathway is one of the most mutated oncogenic pathway, and mutation in PIK3CA occur in 8% to 13% of HNC tumours (LUI *et al.*, 2013). Mutations in PIK3CA gene lead to tumour aggressiveness and have role of target molecules. Great variety of PI3K pathway inhibitors are developed. One of the promising clinically tested PI3K inhibitors is alpelisib, recently approved by FDA, which makes it the first drug for patients with mutated PIK3CA in advanced or metastatic breast cancer (ANDRÉ *et al*., 2149). The highest PIK3CA mutation frequency in HNC is in exon 9 with, followed by exon 20 (FELDMAN *et al.*, 2016). Laryngeal cancer shows prevalence of PIK3 pathway mutations in comparison to other head and neck anatomical sites (LUI *et al.*, 2013). The exon 9 of PIK3CA encodes the helical domain, whereas exon 20 encode kinase domain of the p110α subunit. The mutations in codon 542 or 545 could decrease the inhibitory effect of p85 on p110α leading to increased PI3K activity and enhanced downstream signaling elements, including AKT (KANG *et al.*, 2005). Mutated PIK3CA was found in 14% of our cases, with three "hot spot" mutations in exon 9 (p.Glu542Lys, p.Glu545Gln and p.Glu545Lys) and another two in exon 20, one of them unpublished frameshift p.Pro1011fsTer16. Mutations in PIK3CA gene are associated with poor prognosis in HNC and deregulated PIK3CA may exhibit increased sensitivity to dual PI3K/mTOR inhibition, but development targeted therapy is challenging (LUI *et al.*, 2013).

The fifths most frequently mutated gene was FBXW7, which is a tumour suppressor gene, encoding the ubiquitin ligase protein complex F-box, found mutated in multiple tumours. It has been demonstrated that somatic mutations in FBXW7 occur together with other molecular alterations in patients with advanced cancer (JARDIM *et al.*, 2014). Mutations in FBXW7 were reported in 5% of HNC (AGRAWAL *et al.*, 2011). In our study, we identified that 8.8% of the patients that are HPV negative harbour mutations of this gene. It is known that FBXW7 has a critical role as a tumour suppressor in cancerogenesis, resulting in blocking and further activation of NOTCH1 signaling (DALLOL *et al.*, 2016).

Activating mutations in the oncogenic HRAS gene may have a functional link to the apoptotic pathway, as observed in colorectal cancers (STRANSKY *et al*., 2011). In HNC, HRAS mutations occur in 5% of the samples, and hotspot mutations (G12, G13, Q61), reduce GTPase activity, allowing HRAS to remain in the active state (STRANSKY *et al.*, 2011; AGRAWAL *et al*., 2011). Activated HRAS also facilitates signaling through the MEK/ERK pathway, which is known to cross talk with the PI3K/Akt pathway, and may contribute to PIK3CA-inhibitor resistance through mTOR activation (VANDER BROEK *et al*., 2015). From the RAS family members in HNC, HRAS is the more often mutated gene, which does not support previously published data, where KRAS is the predominantly mutated (PATERSON *et al.*, 1996). We detect two hotspot HRAS mutations in three patients (p.Gly12Ser and p.Gly13Arg), one pathogenic variant in KRAS (p.Gly12Cys), which were related to anti-EGFR therapy (ROTHENBERG *et al.*,

2005). One new variant in NRAS (p.Arg41Gly) is found as well, not published until now. Unfortunately, there are no successful strategies to directly target RAS despite significant drug development efforts (RIZZO *et al*., 2015).

An interesting finding is that in four patients we did not find any pathogenic variants. They belong to the subgroups of glottic (A20) and supraglottic (B2, B10 and B14). In addition, 8 out of 27 patients in the glottis, four patients in the supraglottis and one patient in the subglottis groups showed just TP53 mutation. Previously is published that CNVs are enriched in HNC (CHAU *et al*., 2008; MANTEROLA *et al*., 2018), and major epigenetic changes including methylation imbalance, histone modification, and aberrant non-coding RNA expression could play a role in the development of LSCC (WONG *et al*., 2012). We suggest that more extensive whole exome studies of SNV and CNVs, as well as epigenetic modifications are needed in order to find the driver mutations in this subgroup.

In addition to the pathogenic variants several polymorphisms, previously associated with increased risk, specific tumour characteristics or drug response were found. The rs1042522 polymorphism in TP53 is well studied, with the two alleles encoding Pro72 or Arg72. It is suggested that the Pro72 allele could be more efficient in causing cell cycle arrest, whereas the Arg72 allele is shown to be more efficient in inducing apoptotic response (FERNÁNDEZ-MATEOS *et al.*, 2019). Pro72 appears to be significantly associated with oral squamous cancer and esophageal squamous cancer (ADDURI *et al*., 2014), but little is known about an association with LSCC development. The p.Pro72Arg polymorphism is associated with cancer development and toxicity risks following platinum-based chemotherapy treatment in advanced NSCLC patients, but not with the response to first line platinum based chemotherapy (ZHENG *et al.*, 2014). Moreover, Pro72 homozygous patients show poorer survival rates in comparison to Arg72 homozygotes or heterozygotes (BOLDRINI *et al*., 2008). The additional study of rs1042522 in LSCC could be beneficial for treatment opportunities and patients prediction.

The other neutral variant, rs1870377 (c.1416A>T; p.Gln472His) in the KDR gene (also known as VEGFR2) was previously associated with increased phosphorylation of KDR, which leads to increased angiogenesis, tumour density and shorter PFS (and less so with OS) in the present study population (SILVA *et al.*, 2016). Having a variant in this SNP has also been shown to increased cancer susceptibility. The effect of enhanced tumour angiogenesis may be stronger in terms of increased micro-vessel density and nutrient supply as compared with improved accessibility for drug therapy (imatinib) (RAVEGNINI *et al.*, 2017).

The polymorphism rs3822214 (c.1621A>C; p.Met541Leu) in exon 10 in the KIT gene was previously related with rapid progression, poor prognosis, aggressive metastasis and resistance to imatinib in cancer disease (YIM *et al*., 2018). p.Met541Leu was observed in 9 (16%) of the studied LSCC patients and six of them were positive for nodal metastasis at time of surgery.

Although, these three variants are not pathogenic, their association with specific tumour characteristics could be promising for using them as biomarkers for prediction and prognosis. However, the relative risks associated with them are difficult to estimate and larger studies are needed.

Our study has some limitations. The sample size of the analyzed samples, as well as subgroups is limited and does not allow us make precise conclusions about the exact differences

in mutation landscape between supraglottis, glottis and subglottis. In addition, the number of genes included is also limited to 48, selected based on the existing knowledge about major cancer related pathways. This hampers the ability to discover new, specific for the laryngeal cancer aberrations. However, it has been previously shown that even targeted NGS panels could be successfully incorporated into the clinical practice for patients with HNC, facilitating the stratification of patients for targeted therapy and inclusion in clinical trials (Chau *et al*., 2016).

A comprehensive study of all changes on genomic, transcriptomic and epigenomic level, as well as non-coding RNA analysis is needed to get the complete picture and gain more profound knowledge about the molecular mechanisms of laryngeal cancerogenesis.

CONCLUSION

In conclusion, this study provides detailed somatic mutational analysis with targeted NGS panel in 57 advanced HPV-negative LSCC tumour samples in Bulgarian patients. Pathogenic variants in 27 cancer-associated genes were found and most frequently mutated genes were TP53, MET, CDKN2A, PIK3CA and FBXW7. We suggest that in comparison to other HNC subtypes, NOTCH1 mutations occurred rarely in LSCC patients, and defected TP53, MET and FBXW7 genes were more common more often in LSCC. Moreover, three main laryngeal locations: supraglottis, glottis and subglottis showed slightly different mutational profile from each other. We can hypothesize that LSCC mutation profile and features might differ from other HNCs. Our results enlarge the LSCC mutational spectrum with 13 new variants with pathogenic prediction. Moreover, we found directly targetable mutations with implication in clinical practice for therapy selection in the future. High risk TP53 mutations alone or in combination with RAS mutation are related with reduced cisplatin based therapy sensitivity and worse survival outcome. The MET "driver mutation" p.Thr1010Ile and the family associated p.Asn375Ser lead to activation of the MET signaling pathway, and could be potential biomarkers for innovative targeted therapy in LSCC. The development and implementation of NGS targeted sequencing in the routine laryngeal screening practice will improve the potential of finding novel therapeutic opportunities in advanced HPV-negative LSCC.

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PATOGENE SOMATSKE ALTERACIJE U HPV-NEGATIVNOJ ĆELIJI KOD KARCINOMA GRKLJANA OTKRIVENE CILJANIM SEKVENCIONIRANJEM NOVE GENERACIJE

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Izvod

Tokom poslednje decenije, sekvencioniranje nove generacije (NGS) postalo je glavno sredstvo za otkrivanje somatskih mutacija u karcinomu, ali podaci o mutacijama kod pločastih ćelija karcinoma grkljana još uvek su malobrojni. Cilj ove studije je analiza profila mutacije ključnih onkogena i gena za supresiju tumora u uznapredovalom HPV-negativnom karcinomu pločastih ćelija grkljana. Ukupno je uključeno 57 bugarskih bolesnika s LSCC-om. DNK je izolovana iz sveže smrznutih tkiva. Ciljani NGS je primenjen korišćenjem *TruSeq Amplicon Panel* na *Illumina* platformi, a podaci su analizirani *VarSeq* softverom. Rezultati su otkrili ukupno 92 poznate patogene i verovatno patogene varijante u 27 gena povezanih sa tumorima. Predviđeno je da trinaest novih varijanti bude patogeno sa četiri ili više programa predviđanja. Najčešći mutirani gen bio je TP53, sa mutacijama kod 84,2%, zatim MET kod 19,3%, CDKN2A kod 15,8%, PIK3CA kod 14% i FBKSV7 kod 8,8% pacijenata. Zanimljivo je da su kod osam supraglotskih LSCC pacijenata pronađene dve mutacije TP53, a kod jednog subglotičnog LSCC pacijenta - tri TP53 mutacije. Prvi put je analiziran mutacijski spektar tri LSCC pod-lokacije i supraglottis LSCC pokazao je više mutiranih gena (n = 20) u poređenju sa tumorima glottis (n = 10) i subglottis (n = 12). U 9% uzoraka pronašli smo kombinovane mutacije visokog rizika TP53 i RAS, prethodno povezane sa lošim ukupnim preživljavanjem i otpornošću na lekove. Analiza je pokazala da NOTCH1 mutacije nisu uobičajene za LSCC u poređenju s drugim HNC lokusima. Pored toga, pronašli smo tri uobičajena polimorfizma u genima TP53 (p.Pro72Arg), KDR (p.Gln472His) i KIT (p.Met541Leu), snažno povezanim sa angiogenezom, lošom prognozom i metastazama i osetljivošću na lekove kod bolesti raka. Može se zaključiti da NGS ciljano sekvenciranje omogućava otkrivanje novih mutacija u ključnim genima relevantnim za LSCC, od kojih bi neke mogle biti korisne u odabiru ili razvoju preciznijih LSCC pristupa u budućnosti.

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