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RESEARCH

# Mutational Status of *CDKN2A* and *TP53* Genes in Laryngeal Squamous Cell Carcinoma

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**Abstract** Laryngeal squamous cell carcinoma (LSCC) is the second most common tumour of the head and neck. It is characterized by frequent aberrations in two cell-cycle regulators—*CDKN2A* and *TP53*. However, LSCC has been often studied as a part of the group of head and neck cancers and not as an individual entity. In the current study we aimed to examine mutation status of *CDKN2A* and *TP53* genes in 108 LSCC patients. DNA was extracted from fresh-frozen tumour tissues; exons 1–3 of *CDKN2A* and exons 5–8 of *TP53* were screened for mutations by direct sequencing. Genetic aberrations in *CDKN2A* were found in 16 (14.2 %) and those in *TP53*—in 56/108 (51.9 %) tumours. Seven mutations (two insertions, three deletions, one missense and one silent) detected in *CDKN2A* were not described previously. Also, we found seven novel deletions and a novel indel in *TP53*. No significant associations with clinical features were found. However, *TP53* mutations were predominantly observed in smokers with advanced stage tumours. Screening for genetic aberrations in a defined group of LSCC contributes to the knowledge about laryngeal carcinogenesis. Further investigations are required to confirm the observed trends in associations with clinical features.

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## Introduction

Laryngeal squamous cell carcinoma (LSCC) belongs to the group of Head and neck squamous cell carcinomas (HNSCC). HNSCC is the sixth most common tumour type worldwide with about 600 000 new cases registered each year and almost 50 % mortality rate [1]. Despite all efforts in improving diagnostics and treatment, most patients are still diagnosed at an advanced stage of the disease, which correlates with poor survival [1, 2].

LSCC is known to progress through a series of clinical and histological stages [3]. The alterations, leading to the development of clonal population of transformed epithelial cells, most often arise in genes *TP53*, cyclin-dependent kinase inhibitor 16 (*CDKN2A*), cyclin D1 (*CCND1*) and epidermal growth factor receptor (*EGFR*) [1, 4–6]. These include mutations as well as loss of heterozygosity, deletion/ amplification or hypermethylation. The first two genes—*CKDN2A* and *TP53* are also targets of HPV (human papilloma virus) and thus, rarely mutated in HPV-caused LSCC [7]. However, the information about genetic aberrations in laryngeal carcinomas is still insufficient, as they are usually studied as a part of head and neck malignancies.

The *CDKN2A* gene, mapped to the 9p21 region, is thought to be involved in early stages of LSCC development [8]. It is affected in up to 80 % of HNSCC—often deleted, hypermethylated, or much rarely—mutated [9–12]. As a result of alternative splicing, *CDKN2A* gene encodes two distinct protein products, which are functionally related: $p16^{INK4A}$ and $p14^{ARF}$ [13]. P16 specifically binds CDK4/CDK6 thus inhibiting catalytic activity of CDK/Cyclin D1 complexes, necessary for Rb1 phosphorylation. In this way it inhibits cell

cycle progression from the G1 to the S phase [14]. $P14^{ARF}$ plays a key role in the cell cycle arrest, promoting p53 stability and subsequent transactivation of its target genes.

Located in 17p13 chromosome region, *TP53* is often inactivated at the time of transition from pre-invasive to invasive state of LSCC [15]. In response to various stresses, p53 expression and accumulation are favoured, repressing or predominantly activating target genes responsible for DNA repair, apoptosis and cell cycle arrest. *TP53* gene is affected in up to 60–80 % of HNSCC cases [1, 16]. Approximately 75 % of the mutations are missense, mainly in the DNA-binding domain of p53, encoded by exons 5–8 [17].

Because of the significance of *TP53* and *CDKN2A* in proliferation and development, the tight regulation interplay between them, and the fact that in many tumour types, including LSCC, these two genes are frequently altered, in the current study we analyzed their mutational status in a group of patients with LSCC.

## Materials and Methods

### Patients and Samples

A hundred and eight specimens from patients with histologically confirmed LSCC, but no chemo- and/or radiotherapy prior to surgery, were selected from the LSCC samples collected between 2009 and 2012 in the Department of Otorhinolaryngology, Clinic of Otorhinolaryngology, University Multiprofile Hospital for Active Treatment "Tsaritsa Yoanna—ISUL" Sofia, Bulgaria. The tumour tissues were frozen in liquid nitrogen and stored at −80 °C. All samples were confirmed to be HPV-negative. Epidemiological and clinical characteristics of the patients were gathered as well. The study was approved by the Ethical Committee of Medical University—Sofia and written informed consent was signed by every patient.

### Tumour DNA Extraction

The DNA extraction from tumour tissues was performed with QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the standard protocol provided by the manufacturer. The quality and the quantity of the extracted DNA were assessed using horizontal agarose gel electrophoresis and NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), respectively.

### PCR Amplification and Sequencing

The PCR reactions were carried out in a total reaction volume of 10 μl containing: 30 ng genomic DNA, 0.8 μM of each primers and 1x Red Taq DNA polymerase MasterMix (VWR, Radnor, PA). The sequences of the primers were given in Table 1. While all three exons of *CDKN2A* were examined, only the hot-spot ones, exon 5-to-8 of *TP53* were analyzed. The PCR profile consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 54–70 °C for 30 s and 72 °C for 40 s; and a final elongation step at 72 °C for 10 min.

Direct sequencing was performed as described elsewhere [18]. Briefly, 1.5 μl amplification product were purified with ExoSAP mix, followed by a sequencing reaction using Big Dye® Terminator kit v3.1, (Applied Biosystems, Foster city, CA). The products were then loaded on ABI 3130xl Sequence Genetic Analyzer (Applied Biosystems, Foster city, CA) according to the supplier's protocol. The sequence electropherograms were analysed with Finch TV ver.1.0.4. (Geospiza,Inc.) and CLC Main Workbench 6 (CLC bio).

**Table 1** Sequences of the primers used for the amplification and direct sequencing of *CDKN2A* and *TP53* genes and sizes of the amplified products

| Primer | | Sequence | Size (bp) |
|---|---|---|---|
| CDKN2A_ex1 | F | 5′ CGCCAGCACCGGAGGA AGAAAGA 3′ | 432 |
| | R | 5′ TCGCCAGGAGGAGGTCTG TGATTA 3′ | |
| CDKN2A_ex2 | F1 | 5′ ACAAGCTTCCTTTCCGTC ATGCCG 3′ | 244 |
| | R1 | 5′ CCAGGCATCGCGCACGTC CA 3′ | |
| | F2 | 5′ TTCCTGGACACGCTGGTG GT 3′ | 242 |
| | R2 | 5′ TCTGAGCTTTGGAAGCTCTC AG 3′ | |
| CDKN2A_ex3 | F | 5′ CCGGTAGGGACGGCAAGA GA 3′ | 169 |
| | R | 5′ CTGTAGGACCTTCGGTGACTGA TGA 3′ | |
| TP53_ex5 | F | 5′ CTTTGCTGCCGTCTTCCA GTT 3′ | 334 |
| | R | 5′ TCAGTGAGGAATCAGAGG CCT 3′ | |
| TP53_ex6 | F | 5′ CTGGGGCTGGAGAGACGA CA 3′ | 257 |
| | R | 5′ CTGCTCACCTGGAGGGCC ACT 3′ | |
| TP53_ex7 | F | 5′ CCCCTGCTTGCCACAG GT 3′ | 240 |
| | R | 5′ GGTCAGAGGCAAGCAG AGG 3′ | |
| TP53_ex8 | F | 5′ ATGGGACAGGTAGGACCT GA 3′ | 259 |
| | R | 5′ GTGAATCTGAGGCATAAC TGC 3′ | |

### Statistical Analysis

Statistical analysis of the data was performed using Statistical Package for Social Sciences (SPSS) ver.17.0 (SPSS Inc., Chicago, IL, USA). P value below 0.05 was accepted as statistically significant.

## Results

### Alterations in *CDKN2A* Gene

In our group of 108 LSCC samples 15 genetic alterations were found in exon 1 and 2 of *CDKN2A* gene—Table 2. They were present in 16 patients (14.2 %) and included 6 frameshift, 4 nonsense, 3 missense, 1 silent mutation and 1 SNP. Excluding the SNP, the silent mutation and the one in codon 148, which is known as a polymorphism [26], the others were found in 11/108 (10.2 %) patients. Most of the mutations—10 out of 14, were in exon 2 of *CDKN2A* gene. Seven of them were point mutations, while the other 3 were deletions. In exon 1 of the gene no deletions were found, but 2 insertions and 2 point mutations were detected. One patient—case 77, had a mutation in both exons. Another one—case 63, carried two mutations in exon 2 of the gene. The polymorphism c.442G>A was detected in four patients.

When exon 3 of the *CDKN2A* gene was tested, a SNP was found—rs11515, in the 3'UTR region of *CDKN2A*. Only one patient in our group had the rare GG (1 %) genotype, 36 patients were heterozygous with GC (33.3 %) genotype and the other 71 patients were homozygous with CC (65.7 %) genotype.

After search in the literature and databases (HGMD, COSMIC, dbSNP) we found out that seven of *CDKN2A* aberrations have not been published by now—Table 2. These include the two insertions in exon 1, three deletions and two point mutations in exon 2. The effect of the mutation c.269 T > C F90S was accessed by two online effect-predicting softwares—it was classified as "Probably damaging" with a score rate of 1.0 using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and as "disease-causing" using the SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/).

### *TP53* Genetic Aberrations

Forty-eight *TP53* alternations were detected in 56/108 (51.9 %) patients with LSCC—Table 3. These included 5 SNP; therefore, *TP53* mutations were 43, found in 47 (43.5 %) patients. Thirty-one out of 43 (72.1 %) were point mutations. Twenty two (71 %) of them were missense mutations, predominantly affecting exon 5 of *TP53* and nine (29 %) were nonsense mutations, evenly distributed in all four studied exons. No silent mutations were found.

Six LSCC patients suffered alternation events in more than one of the exons examined. Four of the missense mutations were detected in more than one patient, and three of them were the most prevalent for *TP53* gene G>T transversions, leading to the following aminoacid changes: R175H, C176F and R273L. Another two mutations, each of which affecting two patients, were as followed: a transition at the beginning of

**Table 2** *CDKN2A* genetic alternations found in LSCC patients

| Exon | Genetic aberration | AA change | Sample | Effect | Published/Reference |
|---|---|---|---|---|---|
| 1 | c.1-34 g>t | – | 77 | FS | Yes [19] |
| 1 | c.34ins10bp[a] | – | 24 | FS | No |
| 1 | c.35C>A | p.S12Stop | 97 | N | Yes [20] |
| 1 | c.58insA[a] | – | 2 | FS | No |
| 1 | c.150+37 g>c | – | 16 | SNP | dbSNP rs45456595 |
| 2 | c.181G>T | p.E61Stop | 39 | N | Yes [21] |
| 2 | c.205G>T | p.E69Stop | 83 | N | Yes [22] |
| 2 | c.216C>A | p.C72Stop | 48 | N | Yes [23] |
| 2 | c.243C>T[a] | p.P81P | 64 | S | No |
| 2 | c.260_7del8bp[a] | – | 63 | FS | No |
| 2 | c.269 T>C[a] | p.F90S | 63 | M | No |
| 2 | c.342delC[a] | – | 93 | FS | No |
| 2 | c.343G>T | p.V115L | 27 | M | Yes [24] |
| 2 | c.441delC[a] | – | 46 | FS | No |
| 2 | c.442G>A | p.A148T | 53, 73, 77, 108 | M | Yes [25] |
| 3 | c.471+29 g>c | – | 1–108 w/o 77 | SNP | dbSNP rs11515 |

*M* missense, *N* nonsense, *S* silent, *FS* frameshift mutation, *SNP* single nucleotide polymorphism

[a] Novel aberrations in the gene

**Table 3** *TP53* genetic alternations in LSCC patients

| Exon | Genetic aberration | AA change | Patient | Effect | Published/Reference |
|---|---|---|---|---|---|
| 5 | c.392A>T | p.N131I | 103 | M | Yes [27] |
| 5 | c.394A>G | p.K132E | 62 | M | Yes [28] |
| 5 | c.406_9del4bp[a] | – | 32 | FS | No |
| 5 | c.406C>T | p.Q136Stop | 83 | N | Yes [29] |
| 5 | c.415A>T | p.K139Stop | 14 | N | Yes [30] |
| 5 | c.464C>A | p.T155N | 24 | M | Yes [31] |
| 5 | c.466_9del4bp /insTCC[a] | – | 48 | FS | No |
| 5 | c.468_9del2bp | – | 20 | FS | Yes [32] |
| 5 | c.469G>T | p.V157F | 37 | M | Yes [33] |
| 5 | c.473G>T | p.R158L | 102 | M | Yes [34] |
| 5 | c.474_506del33bp[a] | – | 78 | FS | No |
| 5 | c.487 T>C | p.Y163H | 64 | M | Yes [35] |
| 5 | c.488A>G | p.Y163C | 39, 97 | M | Yes [36] |
| 5 | c.503_55del53bp[a] | – | 51 | FS | No |
| 5 | c.524G>A | p.R175H | 13, 46 | M | Yes [37] |
| 5 | c.527G>T | p.C176F | 4, 29 | M | Yes [38] |
| 5 | c.546C>A | p.C182Stop | 84 | N | Yes [39] |
| 6 | c.578A>G | p.H193R | 76 | M | Yes [40] |
| 6 | c.601_42del42bp[a] | – | 60 | FS | No |
| 6 | c.610G>T | p.E204Stop | 40 | N | Yes [41] |
| 6 | c.614A>G | p.Y205C | 54 | M | Yes [42] |
| 6 | c.637C>T | p.R213Stop | 26 | N | Yes [43] |
| 6 | c.638G>T | p.R213L | 69 | M | Yes [44] |
| 6 | c.639A>G | p.R213R | 11, 19, 20, 53, 55, 80 | SNP | dbSNP rs1800372 |
| 6 | c.659A>G | p.Y220C | 25 | M | Yes [45] |
| 6 | c.672+1 g>a | – | 15,86 | Splice site | Yes [46] |
| 6 | c.672+31 a>g | – | 14, 23, 97, 104 | SNP | dbSNP rs34949160 |
| 6 | c.672+48 g>a | – | 66 | SNP | dbSNP rs17884607 |
| 7 | c.673−36 g>c | – | 69 | SNP | dbSNP rs17880604 |
| 7 | c.673−19 g>a | – | 28 | 28 | Yes IARC TP53 database |
| 7 | c.680C>A | p.S227Y | 106 | 28 | Yes [47] |
| 7 | c.706 T>A | p.Y236N | 72 | M | Yes [48] |
| 7 | c.715A>G | p.N239D | 31 | M | Yes [49] |
| 7 | c.721_6del 6bp[a] | – | 96 | FS | No |
| 7 | c.729_730GG>TT | p.M243_G244>IC | 56 | M | Yes [50] |
| 7 | c.734G>T | p.G245V | 52 | M | Yes [51] |
| 7 | c.742C>T | p.R248W | 2 | M | Yes [52] |
| 7 | c.782_782+18 del19bp[a] | – | 52 | FS | No |
| 8 | c.796G>T | p.G266Stop | 106 | N | Yes [53] |
| 8 | c.818G>T | p.R273L | 58, 94 | M | Yes [54] |
| 8 | c.824G>T | p.C275F | 89 | M | Yes [55] |
| 8 | c.844C>T | p.R282W | 42 | M | Yes [56] |
| 8 | c.852_63del12bp[a] | – | 75 | FS | No |
| 8 | c.853delG | – | 36 | FS | Yes [57] |
| 8 | c.871A>T | p.K291Stop | 28 | N | Yes [58] |

**Table 3** (continued)

| Exon | Genetic aberration | AA change | Patient | Effect | Published/Reference |
|---|---|---|---|---|---|
| 8 | c.880G>T | p.E294Stop | 18 | N | Yes [59] |
| 8 | c.880delG | – | 45 | FS | Yes [60] |
| 8 | c.892G>T | p.E298Stop | 22, 93 | N | Yes [61] |

*M* missense, *N* nonsense, *S* silent, *FS* frameshift mutation, *SNP* single nucleotide polymorphism

[a] Novel aberrations in the gene

intron 6 with impact on splicing and a nonsense mutation in exon 8.

Except for the point mutations, ten deletions and one insertion/deletion were found. Three of them have already been published—Table 3. However, we detected eight novel aberrations.

Mutations in *TP53* gene were present in 8 (57.1 %) of the patients with mutated *CDKN2A*.

### Mutations and Clinical Features

We also analyzed the clinical characteristics of the patients with mutated *CDKN2A* and/or *TP53* genes—Table 4. Probably due to the small number of patients in some of the groups, no statistically significant differences were found (all $p>0.05$, data not shown).

While there were only small variations in the group of *CDKN2A* mutated patients, few trends were observed for *TP53*. The mutations in this gene were found predominantly in smokers (44.9 % vs. 28.6 % for non-smokers) with advanced carcinoma—T4, stage IV.

## Discussion

In the present study we analyzed mutations in *CDKN2A* and *TP53* genes in 108 LSCC patients. *CDKN2A* was mutated in

**Table 4** Distribution of patients with mutations in *CDKN2A* or *TP53* gene according to the clinical characteristics

| Clinical characteristics | Total | Number of patients with mutations in *CDKN2A* gene (%) | Number of patients with mutations in *TP53* gene (%) |
|---|---|---|---|
| Gender | | | |
| Male | 102 | 14 (13.7 %) | 45 (44.1 %) |
| Female | 6 | 0 | 2 (33.3 %) |
| Age | | | |
| ≤60 | 56 | 7 (12.5 %) | 25 (44.6 %) |
| >60 | 52 | 7 (13.5 %) | 22 (42.3 %) |
| T classification | | | |
| T1-T2 | 9 | 1 (11.1 %) | 1 (11.1 %) |
| T3 | 31 | 4 (12.9 %) | 9 (29 %) |
| T4 | 68 | 9 (13.2 %) | 37 (54.4 %) |
| N classification | | | |
| N0 | 79 | 11 (13.9 %) | 36 (45.6 %) |
| N1-3 | 29 | 3 (10.3 %) | 11 (37.9 %) |
| Stage | | | |
| I–II | 9 | 1 (11.1 %) | 1 (11.1 %) |
| III | 28 | 3 (10.7 %) | 8 (28.6 %) |
| IV | 71 | 10 (14.1 %) | 38 (53.5 %) |
| Smoking history[a] | | | |
| Smoker (present or past) | 89 | 13 (14.6 %) | 40 (44.9 %) |
| Non-smoker | 7 | 1 (14.3 %) | 2 (28.6 %) |
| Concentrated alcohol consumption[a,b] | | | |
| ≤50 ml/day | 25 | 5 (20 %) | 10 (40 %) |
| >50 ml/day | 72 | 9 (12.5 %) | 32 (44.4 %) |

[a] Missing data for some patients

[b] Alcohol ≥30%vol

11 (10.2 %) patients, which is close to the reported by Kiwerska et al. 17 %, where they included polymorphisms as well [62]. The differences might be a result of variations in the features of the analyzed tumour samples like localization or tumour stage. Moreover, the percentages reported about HNSCC vary in a big range – 9–58 % [63, 64]. This data confirmed that LSCC is a carcinoma with its individual features that might differ from those of other cancers in the group of HNSCC and emphasized the importance of studying it as a separate tumour type.

Most of the mutations in *CDKN2A* were found in exon 2 of the gene, which is in consistence with previous studies [26, 62]. Ten mutations affected the aminoacids 12 to 141, which form the ankyrin repeats that are important for binding CDK4/6. The six frameshift and four nonsense aberrations would lead to truncated and most often non-functional protein. Having in mind p16 critical role in the cell cycle regulation, these changes would imminently lead to uncontrolled cell proliferation and possible tumour formation.

Along with already described aberrations we found seven novel ones—Table 2. The three deletions in exon 2 comprised small percentage of all alterations registered, which was in consistency with the information in the database so far [22, 65]. On the contrary to our results, insertions were reported to be a significantly frequent event in this exon of *CDKN2A* (http://www.hgmd.org/). The newly discovered missense mutation in exon 2, c.269 T > C affected the ankyrin repeats and online tools for prediction of its effect confirmed that it was potentially pathogenic.

More than 80 % of all genetic alterations, described in exons 5 to 8 of *TP53*, lead to its overexpression and tumourogenesis [66]. We detected mutations in this region in 47 (43.5 %) patients with LSCC, which confirmed the published data [6, 63, 64, 67]. Also, in accordance with the data a very high alteration rate in the exon 5 of the *TP53* gene was detected [68]. The prevalence of point mutations has been established to be greater than any other alternations, as the data pointed out [69]. Missense mutations were the most common ones, as reported by many authors [70–72]. Deletions were also a common genetic event, which has been suggested to be associated with greater tobacco and alcohol exposure [73–75]. Eight of the alternations detected in *TP53*, have not been described by now and were reported here for the first time, all of them were frameshift mutations, triggering the synthesis of non-functional shorter protein—Table 3.

Exogenous carcinogens such as UV light or BPDE (Benzo (a) pyrene diolepoxide), the later of which is present in tobacco smoke, can cause predominance of G:C > A:T alterations at highly mutable CpG dinucleotides, and explain the high rate of transversion events in cancer types such as lung and laryngeal cancer [59, 76, 77]. Furthermore, it has been reported that the frequency of somatic mutations in the *TP53* gene in smokers was higher than that of non-smokers [78]. In the present study, our data support these reports, as *TP53* mutations were almost twice more in smokers than in non-smokers. In addition, mutations at five hotspot codons (175, 205, 245, 248, and 273) account for most of the *TP53* mutations in head and neck cancers (http://www.iarc.fr/p53 and http://p53.curie.fr). Often these aberrations arise at codons, responsible for essential structural or functional residues, resulting in significant loss of binding and transactivation activities of p53. These included any *TP53* missense mutation affecting residues involved in direct DNA interaction (Asn239, Met243, Arg248, Arg273, Cys275, Arg282, and Arg283) and those at hot spot codons (Arg175, Arg213, Gly245, Arg248, Arg273, and Arg282). Mutation events at Arg248 and Arg273 are classified as "contact", while mutations at residues Arg175 and Arg282, which were also found in our patients, are considered "structural" [67, 79, 80].

Furthermore, DNA mutations in the *TP53* gene, such as R175H and R273H, which are frequently observed in tumours, can lead to the decreased processing of pre-miRNAs by Drosha and decreased levels of mature miRNAs in cells, including miR-16-1, miR-143 and miR-145 [81, 82]. These miRNAs negatively regulate some important regulators of the cell cycle and cell proliferation, such as k-Ras (as a target of miR-143) and CDK6 (as a target of miR-16-1 and miR-145) [81]. The p53 stress-response pathway is heavily interconnected with miRNAs not only because it regulates their expression and processing, but also because *TP53* represents a downstream target of miRNAs [83–87].

The data showed that alternations in *TP53* gene increased with the advancing of tumour stage, which confirmed the hypothesis that *TP53* mutations were likely to occur later in the development of LSCC [15, 59]. On the other hand, only 57 % of *CDKN2A* mutated LSCC had also mutation in *TP53*. A possible explanation is that *TP53* was affected in a different way, by LOH or at transcription level [64]. Moreover, it might be *CDKN2A* that was altered not only by mutations but also by deletion or hypermethylation, as previously reported [12, 64].

All the acquired information from this sequencing analysis might be useful to seek correlations with tumour phenotype, prognosis of treatment, patients outcome and survival period. Patients with mutations in the *TP53* gene in particular have shorter survival or a poor response to treatment (comprehensive list of studies available at http://www.p53.iarc.fr/Somatic.html). Moreover, a number of studies have described specific types of mutations that were associated with a worse prognosis compared with other mutations. This is the case for mutations within the DNA-binding motifs that have been repeatedly associated with shorter survival or poor response to chemotherapy [69]. Further investigations are needed to confirm the clinical significance of aberrations in *CDKN2A* and *TP53* genes in laryngeal carcinoma.

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