P53/MDM2 Co-Expression in Laryngeal Squamous Cell Carcinoma Based on Digital Image Analysis

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Abstract. Background: P53 is a key regulator of genomic stability and function, acting as a tumor suppressor protein. Our aim was to correlate P53 expression with murine double minute 2 (MDM2), a proto-oncogene that interacts with P53 and forms an auto-regulatory pathway, in laryngeal squamous cell carcinoma (LSCC). Materials and Methods: A total of 50 LSCC cases were included in the study. Immunohistochemistry was applied by using antibodies to P53 and MDM2 in the corresponding tissue sections. Protein expression levels for both molecules were measured by implementing a digital image analysis assay (immunostaining intensity levels, densitometric evaluation). Results: Overexpression of P53 protein was observed in 16/50 (32%) LSCC cases, while 22/50 (44%) cases strongly expressed MDM2 protein. Interestingly, in 13/50 (26%) cases, combined overexpression of P53/MDM2 was detected. Overall P53 was strongly positively correlated with MDM2 expression ($p=0.001$). Both P53 and MDM2 overexpression were significantly correlated with advanced stage of LSCC ($p=0.032$ and $p=0.001$, respectively). Additionally, MDM2 was found to be associated with poorer survival of patients ($p=0.046$). Conclusion: Aberrant co-expression of P53 and

MDM2 is associated with advanced stage in LSCC. Furthermore, MDM2 overexpression is a frequent and critical genetic event in LSCC and seems to negatively affect survival.

Head and neck squamous cell carcinomas (HNSCC) represent a family of pathological entities demonstrating increased rates worldwide, due to chronic alcohol and tobacco consumption (1). Additionally, persistent infection with oncogenic high-risk human papillomavirus types has been established as another significant etiological factor for HNSCC development and progression (2). Among HNSCCs, laryngeal squamous cell carcinoma (LSCC) is prominent due to its frequency in this anatomical region. Extensive molecular analyses have shown that carcinogenesis in HNSCC is a multistep process of genomic deregulation based on modified cancer stem cells (3). Normal epithelia accumulate gross chromosomal or specific gene instabilities and these are responsible for their neoplastic and finally malignant transformation (4). Over-activation of oncogenes combined with down-regulation of tumor-suppressor genes transforms a normal cell phenotype to a malignant one. Referring to genes that critically modify the cell micro-environment, P53 is of high significance. P53 is a key regulator of genomic stability and function. The gene is located on the short (p) arm of chromosome 17 at position 13.1 (17p13.1). It encodes a nuclear phosphoprotein with a molecular mass of 53 kDa which acts as a transcription factor negatively regulating cell proliferation. It is also involved in a significant number of cell-signaling pathways including the cell cycle, programmed cell death, and DNA repair (5). Normally, wild-type P53 is expressed at a low level in cells, whereas P53 overexpression due to point-mutations is

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frequently detected by immunohistochemistry (IHC) in a broad spectrum of malignancies with different histogenetic origin, including LSCC (6-8). Additionally, murine double minute 2 (*MDM2*), a proto-oncogene (12q14.3) encoding a nuclear-localized E3 ubiquitin ligase, acts as a major negative regulator in the P53–MDM2 auto-regulatory pathway. MDM2 directly binds to P53, represses its transcriptional activity and promotes its proteasomal degradation (9). Gene amplification is the major mechanism of MDM2 deregulation and its overexpression in breast carcinoma, for example, is correlated with aggressive phenotype. For this reason, novel anti-MDM2 agents have been developed and experimentally applied (10). In the current study, we analyzed P53 and MDM2 at the protein level in LSCC cases in order to determine their co-expression and clinicopathological impact. To our knowledge, this is the first study of P53/MDM2 protein co-expression in HNSCCs, focused especially on LSCC based on a digitized image analysis assay.

Materials and Methods

Study group. For the purposes of our retrospective analytical study, 50 (n=50) archival, formalin-fixed and paraffin-embedded tissue specimens (surgical resections or biopsies) of histologically confirmed primary LSCC were used. The specimens were from 45 males and five female patients, all smokers without a positive DNA test or a clear history of human papillomavirus infection. According to their clinical status, patients were treated by chemotherapy (cisplatin-based), radiotherapy, or both combined. The median survival was 43 months. The hospital Ethics Committee consented to the use of these tissues at the Department of Pathology, Hippocraton Hospital, University of Athens, Athens, Greece for research purposes, according to the World Medical Association Declaration of Helsinki guidelines (2008 revised 2014). Informed consent was obtained for experimentation with human subjects.

The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO) Tumour Classification (11). Demographic and clinicopathological data of the examined cases are demonstrated in Table I.

Antibodies and IHC. Ready-to-use anti-P53 (clone DO7, dilution 1:40; Dako, Glostrup, Denmark) and anti-MDM2 (clone IF2, dilution 1:40; Novocastra, Newcastle upon Tyne, UK) mouse monoclonal antibodies were applied in the corresponding cases. IHC for the marker expression was carried out on 4-µm serial sections of the corresponding tissue blocks. The corresponding slides were deparaffinized and rehydrated. All of them were enzyme digested for 10 min at 37°C. The EnVision™+system (Dako) detection system was used for the following detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using the antibodies at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. The

Table I. Demographic and clinicopathological data of the examined Laryngeal Squamous Cell Carcinoma cases (n=50).

	n (%)
Gender	
Male (median age: 57.5 years)	45 (90%)
Female (median age: 61.5 years)	5 (10%)
Grade	
I	4 (8%)
II	19 (38%)
III	27 (54%)
Stage	
II	4 (8%)
III	25 (50%)
IV	21 (42%)
Anatomic location	
Supraglottic	44 (88%)
Transglottic	6 (12%)
Smoking status	
Yes	50 (100%)
No	0 (0%)
Alcohol consumption	
Yes	43 (86%)
No	7 (14%)
Therapeutic strategy	
C	17 (34%)
C-RT	33 (66%)
Survival (months)	
0-16	22 (44%)
>16-30	7 (14%)
>30	21 (42%)

IHC protocol was performed using an automated staining system (I 6000; Biogenex, San Ramon, CA, USA). Breast carcinoma epithelia overexpressing these markers were used as a positive control group. For negative control slides, the primary antibodies were omitted. Predominantly nuclear and slight peri-nuclear cytoplasmic staining was accepted for the markers' positive expression pattern, according to manufacturers' data sheets (Figure 1A and B).

Digital image analysis assay (DIA). P53 and MDM2 protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels (densitometric evaluation). DIA was performed using a semi-automated system (hardware: Microscope CX-31, Olympus, Melville, NY, USA; Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (five optical fields at ×100 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (focal nuclear and peri-nuclear cytoplasmic protein expression). Based on an algorithm, staining of normal tissue sections (control) were measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous greyscale values (0-255) in the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels (Figure 1C and D). Immunostaining intensity values decreasing to 0 represent progressive overexpression of the marker, whereas values increasing to 255 show progressive loss of its staining intensity.

Figure 1. Overexpression of the two markers and digital image analysis in laryngeal squamous cell carcinoma (LSCC): A: P53 and B: murine double minute 2 (MDM2). Note mainly nuclear and cytoplasmic brown staining pattern (diaminobenzidine stain, original magnification: 100×) C: Digital image analysis assay. Red spots represent different expression values of P53-stained nuclei in a case of LSCC. Green loops surrounding red spots represent the final stage of digital analysis providing numerical data (staining intensity values).

Statistical analysis. Descriptive statistics were performed using software package SPSS v25 (IBM, Armonk, NY, USA). Associations between IHC and clinicopathological variables including P53 and MDM2 protein expression levels, gender, tumor grade and stage, anatomical location, and alcohol consumption were extracted by applying Pearson chi-square test. Non-parametric Mann-Whitney *U*-test was used to assess differences in median survival. Two-tailed *p*-values ≤ 0.05 were considered statistically significant.

Results

According to the DIA of protein IHC, overexpression of P53 was observed in 16/50 (32%) LSCC cases, while 22/50 (44%) cases strongly expressed MDM2 protein. Interestingly, in 13/50 (26%) cases a co-overexpression of P53 and MDM2 was detected. Additionally, overall P53 was strongly positively correlated with MDM2 expression ($p=0.001$). Overexpression of P53 and MDM2 were significantly correlated with

advanced stage of LSCC ($p=0.032$, $p=0.001$, respectively). Additionally, MDM2 expression was found to be negatively associated with survival duration ($p=0.046$). No other statistical significance was detected for correlation of P53 and MDM2 with gender ($p=0.712$ and $p=0.259$, respectively), anatomical location ($p=0.524$ and $p=0.253$, respectively), tumor grade ($p=0.115$ and $p=0.528$, respectively), therapeutic regimen ($p=0.194$ and $p=0.622$, respectively) or alcohol consumption ($p=0.136$ and $p=0.115$, respectively). Results and DIA values are described in Table II.

Discussion

The P53 mutational landscape demonstrates insertions, deletions, or simpler base substitutions depending on the histological type of malignancy (12, 13). As well as P53 mutations leading to abnormal protein expression, wild-type

Table II. Expression status of P53 and murine double minute 2 (MDM2) in laryngeal squamous cell carcinoma (LSCC) analyzed cases.

Clinicopathological parameter	n	P53		p-Value	MDM2		p-Value
		OE	LE		OE	LE	
LSCC	50	16/50(32%)	34/50 (68%)		22/50 (44%)	28/50 (56%)	
Gender							
Male	45	14/50 (28%)	31/50 (62%)	0.712	21/50 (42%)	24/50 (48%)	0.259
Female	5	2/50 (4%)	3/50 (6%)		1/50 (2%)	4/50 (8%)	
Grade							
1	4	0/50 (0%)	4/50 (8%)	0.115	1/50 (2%)	3/50 (6%)	0.528
2	19	4/50 (8%)	15/50 (30%)		10/50 (20%)	9/50 (18%)	
3	27	12/50 (24%)	15/50 (30%)		11/50 (22%)	16/50 (32%)	
Stage							
II	4	1/50 (2%)	3/50 (6%)	0.032	2/50 (8%)	12/50 (24%)	0.001
III	25	4/50 (8%)	21/50 (42%)		2/50 (4%)	13/50 (26%)	
IV	21	11/50 (8%)	10/50 (20%)		18/50 (36%)	3/50 (6%)	
Survival (months)							
0-16	22	10/50 (20%)	12/50 (24%)	0.154	13/50 (26%)	9/50 (18%)	0.046
>16-30	7	1/50 (2%)	6/50 (12%)		2/50 (4%)	5/50 (10%)	
>30	21	5/50 (10%)	16/50 (32%)		7/50 (14%)	14/50 (28%)	

P53 OE: P53 overexpression, staining intensity values ≤ 131 (spectrum between 79 and 131), LE: P53 low expression values between 142 and 169; MDM2 OE: MDM2 overexpression staining intensity values ≤ 128 (spectrum between 71 and 128), LE: MDM2 low expression values >139 (spectrum between 139 and 172).

P53 is influenced by two main inhibitors: MDM2 and MDM4. The role of MDM4 is critical because it inhibits the transcriptional activity of P53, enhancing also the ability of MDM2 to target P53 for degradation (14). Overexpression of these molecules is mediated mainly by gene amplification in a broad spectrum of solid malignancies, including LSCC (15-17). Another epigenetic mechanism of deregulation of the P53/MDM2 auto-regulatory pathway is the aberrant alternative splicing of mRNA precursors. This process leads to abnormal protein expression that promotes cell growth, local invasion, and metastasis by enhancing oncogenes and down-regulates suppressor genes (18). Additionally, the role of post-transcriptional regulators, termed microRNAs, seems to be significant for regulating the P53/MDM2 dependent pathway. In particular, positive feedback loops involving micro-RNAs such as *miR-192*, *miR-34a*, and *miR-29a* have been already identified in genetic signatures of LSCC (19).

In the current study, we analyzed P53/MDM2 complex at the protein expression level in LSCC tissues. A significant subset of the examined tumors demonstrated co-overexpression, whereas in the rest, elevated MDM2 expression inversely correlated with P53 level. Interestingly, overexpression of the two molecules was significantly correlated with an aggressive phenotype (advanced stage) in the examined cases. Furthermore, MDM2 overactivation was found to be associated with reduced survival of patients. Similarly, other studies have shown that overexpression of mutated P53 is frequently detected in a significant proportion of LSCC tissues (20, 21). Another study group investigated the

potential role of P53 and B-cell lymphoma 2 (*BCL2*) (gene locus: 18q21.33), an anti-apoptotic factor suppressing and blocking apoptotic signal transduction, as potential reliable molecular markers for patients with LSCC (22). Based on assays of IHC, polymerase chain reaction, DNA ploidy and S-phase fractions, they concluded that aberrant P53 expression due to mutation should be used as a marker for predicting treatment response rates in patients with LSCC. Concerning natural agents that might be promoters of P53-mediated apoptotic activity in LSCC, a study group proposed *Boschniakia rossica* polysaccharide (BRP). Treating Hep2 laryngeal carcinoma cells with BPR at different concentrations they observed that a high apoptotic rate was established due to P53 and enhancement of caspase complex (especially caspase-3) (23). Additionally, another molecular study showed that induction of P53 activity based on *miR-30b* overexpression enhanced adenovirus-mediated P53 cancer gene therapy for patients with LSCC (24). Regarding MDM2 gene up-regulation in LSCC, interesting molecular data have been derived by exploring the role of its specific polymorphisms. One study group showed that MDM2 rs769412 and rs937283 polymorphisms might be used as genetic markers for increased laryngeal carcinoma risk, especially in cases characterized by chronic alcohol consumption (25). Similarly, another genetic study focused on the role of MDM2 -309 T/G single nucleotide polymorphism in a specific population (Chinese Han) analyzing samples by applying pyrosequencing technique. They concluded that the majority of patients with LSCC MDM2 -309TG genotype demonstrated better phenotype (low

stage and metastatic potential) compared to those with TT, characterized also by a reduced plasma MDM2 level, which is considered as a favorable prognostic factor (26). Because MDM2 overactivation negatively modifies the biological behavior of this malignancy, and the response rate to chemoradiotherapy regimens, it should potentially be considered as a marker of LSCC aggressiveness (27, 28). For this reason, small-molecule inhibitors of MDM2, such as nutlin-3, are promising agents for enhancing radiosensitivity of LSCC cells by inducing P53 apoptotic activity (29). Finally, in the current IHC analysis, we applied a DIA protocol for measuring the P53 and MDM2 protein expression levels. This approach provides a totally objective estimation of IHC-based densitometric values in contrast to subjective conventional IHC screening under bright-field microscopy. Especially combined with novel grids in cytological, mainly immunostained, slides, the current technique is a reliable tool for diagnostic and research approaches in the field of accurate, improved evaluation of IHC slides as we have already reported (30, 31).

Conclusion

Overexpression of *MDM2* oncogene, mainly due to gene amplification, is a frequent and critical genetic event in LSCC. In contrast, expression of mutated P53 protein combined with MDM2 leads to an aggressive phenotype (advanced stage). Novel targeted therapeutics should be developed with the aim of blocking MDM2 activity combined with up-regulation of *P53*.

Disclosure

The Authors declare no conflict of interest.

Authors' Contributions

Aristeidis Chrysovergis: Clinical advisor, researcher. Vasileios S. Papanikolaou: Clinical advisor, researcher. Evangelos Tsiambas: Researcher, article writing. Chara Stavraka: Clinical advisor, statistical analysis. Vasileios Ragos: Academic advisor. Dimitrios Peschos: Academic advisor. Amanda Psyrris: Academic advisor. Efthymios Kyrodimos: Academic advisor, article writing. Nicholas Mastronikolis: Case stratification, statistical analysis.

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