

Loss of heterozygosity in tumor-related genes in patients with squamous cell carcinoma of the larynx

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Introduction. Allelic deletion in cancer can be detected by analyzing the loss of heterozygosity (LOH). The aim of our study was to search for LOH in specimens of squamous cell carcinoma of the larynx (SCCL), using markers linked to chosen genes involved in carcinogenesis, such as: *MLH1*, *HPC1*, *MSH2*, *TP53*, the unknown tumor suppressor gene at 8p22, *NM23* (*NM23-H1*, *H2*, *H3*), *MET*, *APC*.

Material. 62 patients with primary SCCL – analyses were performed on DNA isolated from larynx tumor tissues and corresponding peripheral blood lymphocytes.

Methods. PCR reactions and analyses of amplified fragments in ABI-377 were carried out following standard procedures. Genescan and Gentyper software (Applied Biosystems) were used to measure, quantify and compare normal and tumor DNA patterns for each fluorescent marker. An allele ratio of less than or equal to 0.7 was taken to be indicative of LOH.

Results. Specific LOH was observed for markers linked to the following genes: *MLH1* – 3p22 (47.9%), the unknown tumor suppressor gene at 8p22 (37.3%) and *NM23-H1* – 17q21 (21.8%). We observed a positive correlation between lymph node metastases and LOH frequency for *NM23-H1* ($p < 0.05$). An association was also found between the frequency of LOH in *NM23-H2* and both lymph node metastasis *N1* ($p < 0.05$), and advanced stage of the disease ($p < 0.05$).

Conclusion. Our results confirm the observations of other authors, that allelic loss in the following genes: *MLH1*, the unknown tumor suppressor gene at 8p22 and *NM23*, plays an important role in SCCL carcinogenesis.

Badanie utraty heterozygotności w wybranych genach u pacjentów z płaskonabłonkowym rakiem krtani

Wstęp. Alleliczną delecję w guzach nowotworowych można ocenić za pomocą analizy utraty heterozygotyczności (LOH). Celem pracy były badania częstości występowania LOH w płaskonabłonkowym raku krtani (SCCL), przy użyciu markerów sprzężonych z wybranymi genami, zaangażowanymi w proces karcinogenezy, takimi jak: *MLH1*, *HPC1*, *MSH2*, *TP53*, nieznany gen supresorowy (8p22), *NM23* (*NM23-H1*, *H2*, *H3*), *MET*, *APC*.

Materiał. 62 pacjentów z pierwotnym SCCL - badania przeprowadzono na DNA wyizolowanym z tkanek guza krtani i z krwi obwodowej.

Metody. Reakcje PCR i analizę zamplifikowanych fragmentów DNA w ABI-377 wykonano według standardowych procedur. Ocenę i porównanie wyników badań dla par krew i guz, dla każdego markera fluorescencyjnego, przeprowadzono przy użyciu programów Genescan i Genotyper (Applied Biosystems).

Wyniki. Specyficzną LOH obserwowano dla markerów sprzężonych z następującymi genami: *MLH1* - 3p22 (47,9%), nieznany gen supresorowy w 8p22 (37,3%) oraz dla *NM23-H1* - 17q21 (21,8%). Stwierdzono pozytywną korelację między obecnością przerzutów w okolicznych węzłach chłonnych i częstością LOH w *NM23-H1* ($p < 0,05$). Znaleziono związek między częstością LOH w *NM23-H2* a obecnością przerzutów w węzłach chłonnych *N1* ($p < 0,05$) i zaawansowanym stadium choroby nowotworowej ($p < 0,05$).

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Podsumowanie. Wyniki prezentowanych badań potwierdzają wyniki badań innych autorów, że alleliczna utrata w genach: MLH1, nieznanym genie supresorowym (8p22) oraz NM23 odgrywa ważną rolę w karcynogenezie SCCL.

Key words: carcinoma of the larynx, genes, loss of heterozygosity

Słowa kluczowe: rak krtani, geny, utrata heterozygotności

Introduction

Neoplastic development is a complex process resulting from the accumulation of genetic alterations in a single cell. This process leads to disturbances in growth, differentiation, proliferation, and senescence and/or cell death.

Gene mutations and numerical and structural chromosomal alterations in tumors were described. In tumorigenesis the most critical mutations occur in the following groups of genes: tumor suppressor genes, mutator genes and proto-oncogenes. Mutations in proto-oncogenes result in their activation. These mutations are dominant at the cellular level. Mutations in tumor suppressor genes and mutator genes are recessive at the cellular level and therefore two inactivating mutations are necessary for gene inactivation. Mutations in these genes are characteristic for both hereditary (germline mutation) and sporadic (somatic mutations) cancers. The first mutation (germline or somatic) in recessive genes is quite often a point mutation (e.g. frame-shift, non-sense), according to Knudson's two-hit hypothesis. The second hit (always somatic), by which recessive mutations are manifested, is usually a loss of genetic material (microdeletion, loss of a part or the whole of a chromosome) [1]. However, a point mutation or an epigenetic event have also been observed. LOH (loss of heterozygosity) analysis is used to test for allelic deletion [2]. Such analysis uses the polymorphic microsatellite markers that are very frequent in the human genome. Normal and tumor DNA from individual patients are compared with respect to changes in various microsatellite markers. LOH is frequently observed in given markers only for specific types of cancer.

Loss of heterozygosity is defined to be:

- random, when the frequency of LOH is less than 20% at a given locus,
- specific, if it occurs in more than 20% of specimens for a particular marker [3, 4, 5].

LOH for polymorphic DNA markers, observed in DNA isolated from tumors when compared to DNA isolated from matched normal tissue, is a sign of somatic deletion [6].

LOH has been detected in various types of tumors, most often in epithelial cancers such as: colon, renal, breast, lung and bladder cancer.

According to Knudson's hypothesis, loss of heterozygosity plays an important part in tumor initiation. It has also been observed in the early and later stages of carcinogenesis [7, 8].

Cancer of the head and neck accounts for some 2-3% of all malignancies [9]. Most malignant neoplasms in

this region (95%) arise from the surface epithelium and are therefore squamous cell carcinomas (Head and Neck Squamous Cell Carcinoma – HNSCC). The remaining 5% of tumors originate from ducts of the salivary glands. Head and neck squamous cell carcinomas are a heterogenous group of carcinomas, with varied histopathology (e.g. adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma), and different anatomic sites (e.g. the oral cavity including the tongue, the nose and nasal sinuses, the pharynx, larynx and salivary glands).

The growth of SCCL (Squamous Cell Carcinoma of the Larynx) is related to genetic factors, as well as to exposure to biological (e.g. papilloma viruses) and chemical carcinogens (e.g. tobacco, alcohol) [10]. Cancer of the larynx is more frequent in people addicted to tobacco and alcohol and is more common in men. SCCL has also been sporadically observed in people, who neither smoked nor drank alcohol [11].

Genetic studies of HNSCC have revealed frequent chromosomal alterations, mutations in genes critical in carcinogenesis, as well as microsatellite instability (MSI) or LOH in tumor cells [10]. Allelic loss in HNSCC usually affects loci in the following regions: 3p, 8p, 9p, 17p, 18q (Table I) [12-26]. The clinical significance of genetic alterations may be varied, e.g. allelic loss in 8p is

Table I. Loss of heterozygosity (LOH) in head and neck squamous cell carcinoma (HNSCC) – resume of published data

No of cases	LOH	Reference
HNSCC		
81	3p, 9p, 17p	Miracca et al., 2000 [13]
30	3p, 4q, 7q, 9q, 17p, 18q	Ng et al., 2000 [14]
50	22q11.2-q13	Poli-Frederico et al., 2000 [15]
47	9p21	Miracca et al., 1999 [16]
51	8p23	Ishwald et al., 1999 [17]
57	3p21, 9q21	Matsuura et al., 1999 [18]
52	3p, 9p, 17p	Nunn et al., 1999 [19]
36	3p, 9p, 17p	Jin et al., 1999 [20]
30	8p21-22	El-Naggar et al., 1998 [21]
21	9p21	Lydiatt et al., 1998 [22]
67	18q	Pearlstein et al., 1998 [23]
35	8p23, 8p22, 8p12-21	Wu et al., 1997 [24]
SCCL		
19	8p21, 9p21, 17q21	Rizos et al., 1998 [25]
59	8p23	Scholnick et al., 1996 [26]

HNSCC – Head and Neck Squamous Cell Carcinoma
SCCL – Squamous Cell Carcinoma of Larynx

associated with a poor prognosis in patients with supraglottic squamous cell carcinomas, and LOH at 9p loci is associated with the early stages of development of various HNSCC [12, 26]. Loss of heterozygosity in HNSCC at two or more loci seems to be associated with a poor prognosis [3, 12, 27].

However, neither critical genes nor critical regions have been yet determined in the genetic etiology of HNSCC or SCCL. Various models of genetic alterations in HNSCC have recently been developed, such as the model by Califano (genetic progression of HNSCC) [28], Hoglund [29] and Huang [30].

The aim of this study was to investigate which of the chosen genes (important in carcinogenesis) plays a part in the etiology and progression of HNSCC. We analyzed the frequency of LOH in the chosen tumor suppressor genes: *TP53*, *HPC1*, *APC*, the unknown tumor suppressor gene at 8p22, the mutator genes: *MLH1*, *MSH2*, the metastasis gene: *NM23* and the oncogene *MET*.

Material

The study group consisted of 62 patients (56 men and 6 women); mean age 56 years (range: 43-71 years). All of them had primary squamous cell carcinoma of the larynx and were treated between the years 1997 and 2001 at the Department of Otolaryngology of the Medical University in Wrocław. All patients were smokers (10 to 30 cigarettes daily for 20 – 40 years).

The tumors were diagnosed histopathologically as: carcinoma planoepitheliale keratodes – 32 cases, carcinoma planoepitheliale akeratodes – 26 cases and carcinoma planoepitheliale keratoblasticum – 4 cases.

According to the degree of differentiation the tumors were divided into three groups (G -grading): well differentiated carcinomas (G1) – 12 cases, moderately differentiated carcinomas (G2) – 35 cases and poorly differentiated carcinomas (G3) – 15 cases.

According to TNM classification 4 cases were designated as T1; 3 as T2; 29 as T3 and 26 as T4.

Lymph node metastases were observed in 17 patients (8 cases of N1, 8 cases of N2 and 1 case of N3). No distant metastases were found (M0).

At the time of diagnosis in a majority of patients the tumour was already at an advanced stage: stage IVa – 30 cases

(48.4%), stage III – 24 cases (38.7%). The remaining diagnoses were as follows: stage I – 4 cases (6.5%), stage II – 3 cases (4.8%) and stage IVb – 1 case (1.6%).

Methods

Genomic DNA was isolated from peripheral blood lymphocytes and larynx cancer tissue (freshly removed or frozen at -20° C) following standard procedures. To ensure high quality the DNA (3µg) was DOP-amplified (Degenerate Oligonucleotide Primed – PCR) using the NRich™ kit (GENPAK Limited). PCR reactions were carried out with fluorescent markers in a 25 µl solution consisting of: 10 pmol/µl of each of the respective primers (MWG-Biotech AG) (Table II), 15 mM MgCl₂ (Qiagen), 1.25 mM of each dNTP (Boehringer Mannheim), 1 IU/µl RedTaq DNA Polymerase (Sigma), 10xPCR buffer (Sigma), and 100 ng DOP-amplified templates. The thermal profile was as follows: 1 cycle: 95°C for 5 minutes, 36 cycles: 94°C for 30 seconds, 53-61°C for 1 minute, 72°C for 30 seconds, 1 cycle: 72°C for 10 minutes.

Fluorescent PCR products were separated on 2% agarose gel and then pooled and resolved on a 4% polyacrylamide gel supplemented with 7M urea in an ABI-377 automated sequencer. Genescan and Gentyper software (Applied Biosystems) were used to measure, quantify and compare normal and tumor amplicon patterns for each fluorescent marker. The area of the peak for the tumor was compared to the area of the peak for the corresponding normal tissue from each patient by means of their ratio. The peaks produced by the normal DNA sample were used to determine whether the sample was homozygous (only one peak visible) or heterozygous (two peaks visible) (Figure 1a, 1b). Only informative cases (constitutional heterozygotes) were analyzed.

The allele ratio of the areas was calculated as follows: $T1 (N2 / (T2 (N1))$, where N1, N2 are the areas of the shorter and longer allele peaks for the normal sample and T1, T2 are the area of the shorter and longer allele peaks for the tumor sample, respectively [6, 31]. In cases where this was above 1.0, it was converted using the following expression: $T2 (N1 / (T1 (N2))$, to give a result in the range 0.0-1.0.

Up to 30% of the cells in some tumors may represent normal stromal cells (interspersed among the tumor cells). Thus a complete allelic loss in such tumors would give an allelic ratio of approx. 0.7. Hence, this ratio was taken to be indicative of LOH (Figure 1c) [31].

Statistical analysis was carried out using Pearson's test for correlation and Fisher's exact test of independence.

Table II. Characteristics of the microsatellite (dinucleotide) markers

Marker	Chromosome	Gene	Dye (colour)	Size (bp)
D1S2883	1q24	<i>HPC1</i>	FAM (blue)	179-199
D2S123	2p16	<i>MSH2</i>	TET (green)	150-180
D3S1611	3p22	<i>MLH1</i>	TET (green)	184-260
D5S346	5q21	<i>APC</i>	FAM (blue)	107-129
D7S501	7q31	<i>MET</i>	TET (green)	217-233
D8S254	8p22	Unknown tumor suppressor	TET (green)	65-75
NM23 M1	17q21	<i>NM23</i>	FAM (blue)	95-105
NM23 M2	17q21	<i>NM23</i>	TET (green)	176-184
NM23 M3	17q21	<i>NM23</i>	HEX (yellow)	147-171
TP 53	17p13	<i>TP53</i>	HEX (yellow)	106-138

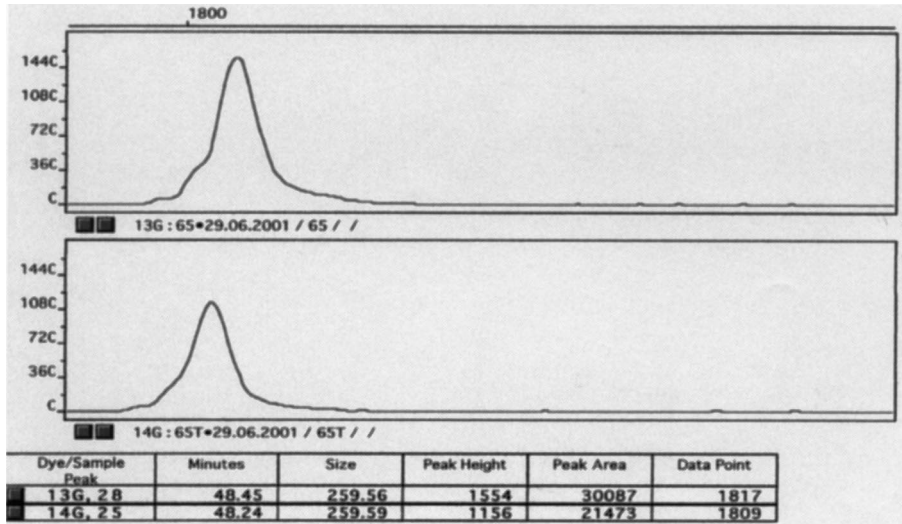


Figure Ia

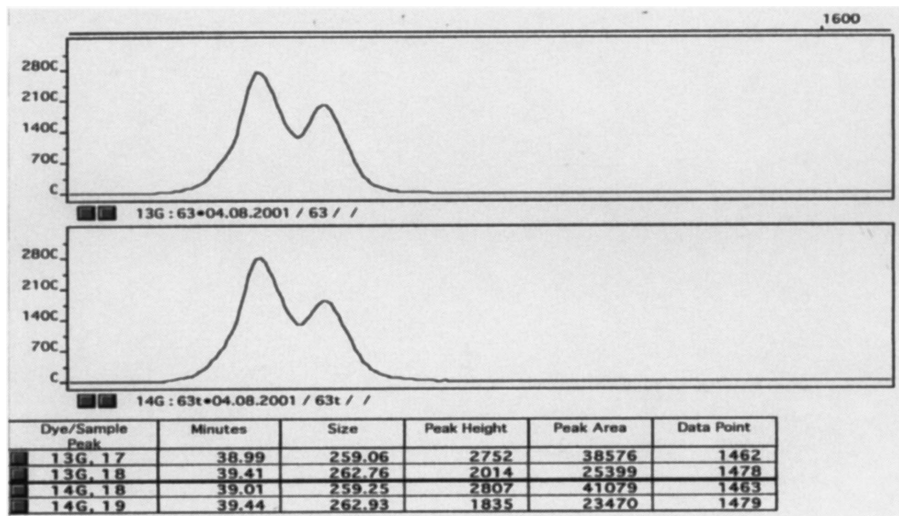


Figure Ib

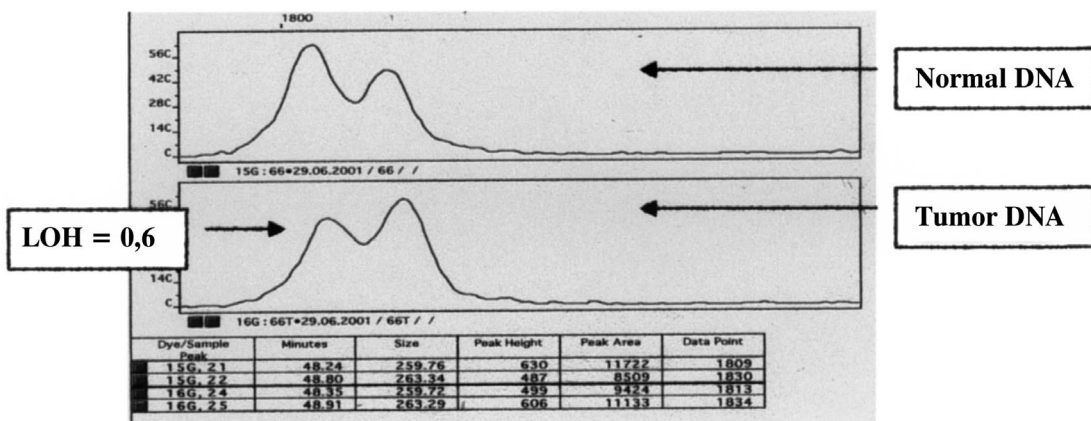


Figure Ic

Figure I. Marker D3S1611
 a) Homozygote
 b) Heterozygote
 c) Loss of heterozygosity (LOH)

Results

LOH analysis was performed for 10 microsatellite markers linked to the following genes:

- 1) tumor suppressor genes: *HPC1* (marker D2S2883), *APC* (D5S346), *TP53* (TP53) and the unknown tumor suppressor gene at 8p22 (D8S254),
- 2) mutator genes: *MLH1* (D3S1611), *MSH2* (D2S123),
- 3) metastasis gene: *NM23* (NM23 M1, M2, M3),
- 4) oncogene: *MET* (D7S501).

The results are summarized in Table III.

Table III. The results of LOH analysis in 62 patients with squamous cell carcinoma of larynx

Gene / chromosomal localisation	Number of informative cases (% of total cases)	LOH (%)
<i>MLH1</i> / 3p22	48 (77.4)	23 (47.9)*
<i>HPC1</i> / 1q24	42 (67.7)	6 (14.2)
<i>APC</i> / 5q21	58 (93.5)	10 (17.2)
Unknown tumor suppressor / 8p22	53 (85.4)	20 (37.7)*
<i>MSH2</i> / 2p16	48 (77.4)	7 (14.5)
<i>MET</i> / 7q31	46 (74.1)	4 (8.6)
<i>TP53</i> / 17p13	49 (79.0)	4 (8.1)
<i>NM23</i> (M1) / 17q21	55 (88.7)	12 (21.8)
<i>NM23</i> (M2) / 17q21	42 (67.7)	5 (11.9)
<i>NM23</i> (M3) / 17q21	51 (82.2)	7 (13.7)

* statistically significant higher frequency of LOH compared to the remaining genes ($p < 0.05$)

For all analyzed genes the results were informative in more than 67% of cases. Specific loss of heterozygosity (LOH frequency $> 20\%$ for the screening locus) was observed for the following genes: *MLH1* – 3p22 (47.9%), the unknown tumor suppressor gene at 8p22 (37.3%) and for *NM23-H1* – 17q21 (21.8%).

The LOH frequency for markers D8S254 (the unknown tumor suppressor gene at 8p22) and D3S1611 (*MLH1*) was significantly higher than for the remaining loci ($p < 0.05$). A positive correlation between lymph node metastases and LOH frequency for *NM23-H1* was observed ($p < 0.05$). An association was found between the frequency of LOH in *NM23-H2* and both lymph node metastasis N1 ($p < 0.05$), and advanced stage of the disease ($p < 0.05$). No statistically significant correlation between the frequency of LOH and any other clinical or histopathological features of the cancers was found.

In the majority of cases specific LOH in *MLH1* (56.5%), the unknown tumor suppressor gene at 8p22 (60%), and *NM23-H1* (58%) were associated with advanced stage of the disease.

LOH at two or more loci were found in 27 of the SCCL (43.5% of the cases). In 13 cases (20.9%) LOH was present at 2 loci, in 6 carcinomas (9.7%) at 3, in 6 cases (9.7%) at 4 and in 2 subjects (3.2%) at 5 loci. Larynx cancers with LOH at ≥ 2 loci were characterized as follows:

- 1) 15 cancers were graded as G2 (55.6%), 6 as G1 (22.2%) and 6 as G3 (22.2%)
- 2) 15 were in stage IVa (55.6%), 8 in stage III (29.6%), 3 in stage I (11.1%) and 1 cancer in stage II (3.7%).

The analysis of association between the frequency of LOH in various loci revealed a significant positive correlation between the incidence of LOH in the following pairs of markers:

NM23 M2 \leftrightarrow NM 23 M3	↓ decreasing value of the correlation coefficient
NM 23 M2 \leftrightarrow D8S254	
D5S346 \leftrightarrow D2S123	
D2S123 \leftrightarrow D8S254	
NM 23 M3 \leftrightarrow D8S254	
D2S123 \leftrightarrow NM23	

Discussion

Loss of heterozygosity in an informative, microsatellite marker linked to the gene indicates a deletion of one of the two alleles of that gene. LOH may be one of the mechanisms of gene inactivation, which is critical in carcinogenesis. LOH in a particular chromosomal region is generally considered to indicate the location of a tumor suppressor gene within the deleted area. LOH is not tantamount to loss of gene expression.

LOH analysis in this study was performed using 10 microsatellite markers linked to the following genes: five tumor suppressor genes, two mutator genes, one metastatic gene and one oncogene. LOH appeared most frequently in markers at the following loci: 3p22, 8p22 and 17q21 (marker *NM23* M1) (with a frequency of $> 20\%$) (Table III). The occurrence of LOH was judged to be random for markers at the following loci: 17p13, 1q24, 2p16, 5q21, 7q31, 17q21 (*NM23* M2, *NM23* M3).

These results are similar to the results obtained in many studies of HNSCC, which have shown that genes in the regions 3p and 8p play a significant role in the development of HNSCC [5, 9, 14, 17, 19, 20, 21, 32]. Bockmuhl et al. postulated that allelic loss in the 3p region is a frequent alteration in head and neck cancers and suggested that tumor suppressor genes located on the short arm of chromosome 3 are connected with the pathogenesis of these cancers [33]. On the basis of comparative genomic hybridization, Kujawski et al. observed deletions in the 3p region in the majority of primary cancer of the larynx. This deletion does not occur in the cells of metastases of these cancers. The authors suggested that losses in 3p form an important event in the early stages of the development of SCCL. An intact chromosome 3 in cancer cells seems to be associated with the advancement of SCCL and promotion of metastasis [32]. Allelic loss at 3p was observed by Grati et al. in more than 50% of cancers of the oral cavity and oropharynx investigated [34]. Mao et al. recorded frequent LOH at the 3p14 and 9p21 loci in premalignant lesions of the oral cavity [35]. LOH in these cases was associated with the development of late-onset HNSCC. These observations support the Califano model, according to

which, specific genetic changes in head and neck cancer occur in a distinct order leading to invasive cancer, with losses at 9p21 or 3p21 being among the earliest detectable events [28].

Allelic loss or deletion in the 3p region has been reported in a variety of neoplasms, such as: esophageal, lung, breast, testis cancers [36, 37].

The 3p region, according to Maestro et al., contains tumor suppressor genes in at least three distinct places (hot spots): 3p14-cen, 3p21.3 and 3p24-pter [38]. One of these genes is *FHIT*, which is mutated in 80% of cell lines of HNSCC [39]. A high incidence of LOH in the region 3p21 was observed by El-Naggar et al. [40] in various HNSCC. However, LOH at 3p13-14 was recorded with a frequency of up to 64% in supraglottic larynx cancer by Scholnick et al. [41].

The mutator gene *MLH1* is located in the 3p22 region. The incidence of LOH for the marker linked to *MLH1* was associated with stage IV of the disease in over 56% of tumors in this study. This observation suggests that *MLH1* plays a significant role in the progression of SCCL.

In this study a high frequency of LOH in 8p22 was observed. The microsatellite marker analyzed is linked to the unknown tumor suppressor gene. LOH at 8p22 was associated with stage IV of the disease in 60 % of cases. Deletions in the short arm of chromosome 8 have been frequently found in HNSCC [25]. Rizos et al. observed loss of heterozygosity in 8p21 in up to 50% of cases of laryngeal carcinomas and revealed that LOH in 8p21, 9p21 and 17q play an important role in the development of SCCL [25]. Sunwoo et al. analyzed the 8p23 region in 150 cases of laryngeal and oral squamous cell carcinomas. They found two distinct deletions in this relatively small region of the chromosome. The authors reported that there was a difference between the incidence of LOH mapped near the telomeric and centromeric end of this region. These data suggest that the high incidence of LOH observed at the 8p23 loci is not a result of the general instability of chromosome ends. The authors suggest that there are two tumor suppressor genes in the 8p23 region [42]. As mentioned above, Scholnick et al. indicated three minimal regions of allelic loss in 8p, mapped to the 8p23, 8p22-23, 8p21 regions and concluded that at least three putative tumor suppressor genes are present in 8p [26]. These results are in agreement with the results of similar studies on cancers of the oral cavity and oropharynx. Wu et al. analyzed deletions within the 8p region in these cancers and described three regions as having a high frequency of LOH: 8p22, 8p23, 8p12-p21.3. According to Wu et al. deletions at 8p form a late event, associated with more aggressive types of cancers [24]. Allelic loss in the 8p region was also reported in a variety of neoplasms, such as: colorectal, non-small lung, hepatocellular, prostate, bladder, breast and pancreatic cancers [21, 42, 43, 44]. These results justify molecular genetic analysis of these regions. The tumor suppressor gene *N33* in 8p22, involved e.g. in the development of prostate cancer, was mapped

by Bova et al. [43]. Fujiwara et al. investigated the region of 8p21.3-p22, which was frequently deleted in sporadic colorectal cancer, hepatocellular and non-lung cancers, and mapped the *PRLTS* gene (PDGF-receptor beta-like tumor suppressor) [45].

A significant correlation between LOH in the 8p region and the advancement of HNSCC was observed by Navroz et al. [5]. El-Naggar et al. recorded a significant association between LOH in 8p in laryngeal and oral squamous cell carcinomas and the early steps of carcinogenesis [21]. Fujiwara et al. suggested that the *PRLTS* gene (8p21.3-p22) is a tumor suppressor gene, which is commonly deleted in sporadic hepatocellular carcinomas, colorectal cancers, and non-small cell lung cancers. The inactivation of the *PRLTS* gene is supposed to play a more significant role in the initiation of carcinogenesis than in its progression [45].

Kujawski et al. analyzed loss of heterozygosity at 8p both in primary laryngeal carcinomas and in lymph node metastases, and observed LOH more often in lymph node metastases, although this difference was not statistically significant [32]. However, according to the model proposed by Nowell describing tumor tissue as a collection of competing subclones, Kujawski et al. reported that loss of heterozygosity in a fragment or whole of a chromosome (particularly at loci in 8p, 9q and 13) in a particular subclone could be an alteration leading to the invasion to adjacent lymph nodes [32, 47]. On the basis of this study and the data presented by Nishizaki et al. in breast cancer, Kujawski et al. suggested that accumulation of further changes is necessary for tumor progression and invasion [32, 47]. An association between the high incidence of LOH at 8p and clinical malignancy of HNSCC was described by El-Naggar et al. [12]. As mentioned above Scholnick et al. [26] observed that allelic loss in 8p23 appears to indicate a poor prognosis for patients with squamous cell carcinoma of the supraglottic larynx. The results of the study by Oba et al. on prostate cancer suggest that deletions in 8p22-p21.3 play an important role in tumor differentiation, while deletions in 8p21.1-p21.2 play a role in the progression of prostate cancer [50]. Miyaki et al. reported a high frequency of allelic loss in 8p22 in the metastases of colon cancer, suggesting that a loss of functioning of genes on chromosome 8 can play a significant role in the progression of colorectal cancer [51].

In the present study three markers (NM23 M1, M2, M3) linked to the *NM23* gene were analyzed. The *NM23* gene is involved in the control of metastasis. LOH occurred with a high frequency for the NM23 M1 marker but not in the NM23 M2 and NM23 M3 markers. The difference in the frequency of LOH for NM23 M1, M2 and M3 probably arises from the differences in the size of the deleted regions.

The incidence of LOH in the NM23 M1 marker correlated positively with lymph node metastasis. Loss of heterozygosity in this marker was associated with stage IV of the disease in 58% of patients. A significant association between LOH in the NM23 M2 marker and

lymph node metastasis N1 and stage IV of the disease was noted.

The data presented by different authors confirm that the 17q21 region plays an important role in the development of HNSCC [53]. Loss of heterozygosity in *NM23-H1* has been observed in other tumors, e.g. squamous cell lung carcinoma, colorectal cancer, gastric cancer and melanoma. The results obtained by Seifert et al. do not show a link between LOH at *NM23* and metastasis in colon and gastric cancer [53].

Basing on the fact that the loss of heterozygosity in a gene is not synonymous to loss of gene expression, many investigators have examined the association of LOH at *NM23* with the expression of *NM23* and tumor development. Bonsar et al. examined squamous cell lung carcinomas (SLC) for LOH and *NM23-H1* gene expression, and concluded that neither LOH of the *NM23-H1* gene nor the intensity of the appropriate proteins are related to the development and progression of SCCL [54]. This observation is in agreement with the conclusion of Pestereli et al. that *NM23* is not associated with progression in larynx cancers [55]. In contradiction to the results of Bonsar and Pestereli, McDonald et al. indicated a negative association between patients' survival times and expression of *NM23-H1* in primary HNSCC [54, 55, 56]. A very interesting observation was described by Miyazaki, who examined the expression of *NM23-H1* and *NM23-H2* genes and suggested that the metastasing process in patients with HNSCC is inhibited only by correct expression of the *NM23-H2* gene [57].

In this study a low frequency of LOH was observed for the markers linked to the following genes: *APC*, *TP53*, *MET*, *HPC1*, *MSH2*. These results do not confirm the data presented in other studies. Ah-See et al. and Uzawa et al. observed a high frequency of LOH in the *APC* gene in HNSCC [4, 58] and suggested that mutations of *APC* are important in the development of HNSCC. Grati et al. noted a high frequency of LOH in the 5q region in squamous cell carcinomas of the oral cavity and oropharynx [34].

Similar to mutations in the *TP53* gene, mutations in the *APC* gene have been described by many authors in various types of tumors, i.e. in cancer of the larynx. This suggests that mutations of *TP53* occur in the early stages of cancer of the larynx and may be an indicator of progression [59, 60]. The different results obtained in other studies may be explained by the fact that *TP53* inactivation in HNSCC is related to point mutations rather than to allelic losses [59].

In this study no significant loss of heterozygosity in the 7q31 region was observed. Matsuura et al. observed a significant loss of heterozygosity at the 7q31 and 9p21 loci in HNSCC. They suggested that genetic changes which give rise to allelic loss happen at multiple sites on the genome and the loss of functioning of 7q31, as well as that of 9p21, may play an important role in the carcinogenesis of head and neck carcinoma, in combination or independently. LOH at 9p21 and 7q31 is associated with

high frequency of recurrent tumors in patients with HNSCC [61].

The *c-MET* oncogene is located in the 7q31 region, which is linked to a microsatellite marker used in the present study. Seruca et al. observed alterations in this gene in up to 50% of well differentiated gastric adenocarcinomas, with a large proportion of these changes being characterized by amplification of the *c-MYC* gene. It is likely that such changes are associated with the stage of the disease and prognosis [62].

In this study a low frequency of LOH was noted for the marker linked to the *HPC1* gene. Latini et al. observed a similar low frequency of LOH in this gene in sporadic prostate cancers [63]. A high frequency of loss of heterozygosity in 1q, together with LOH in 5p, 11p, 17p, 18p and 18q was noted in adenocarcinoma of the cervix *in situ* [64].

In this study a low frequency of LOH in the marker close to loci of the mutator gene *MSH2* was observed. Ransom et al. revealed that poor prognosis in HNSCC is associated with loss of heterozygosity at 2q [65]. A high frequency of LOH in 2q was observed in esophageal squamous cell carcinoma, particularly in the 2q33-q35 region, suggesting the possible location of tumor suppressor genes in this region [36].

Investigations on loss of heterozygosity in various tumors, such as: head and neck carcinomas, colorectal cancers or brain tumors have indicated that LOH in more than 2 loci is significantly associated with a poor prognosis [3]. The results from this study seem to confirm these observations. The analysis of LOH in the investigated loci demonstrated loss of heterozygosity at ≥ 2 loci in 27 of cases (43.5%). Up to 85% of cancers with LOH at more than 2 loci were in advanced stages of the disease (III or IV). This observation suggests that an accumulation of LOH is associated with tumor progression. These results are in agreement with those of other research groups [3, 12, 27].

In conclusion, this study indicates specific loss of heterozygosity occurring in larynx cancers in the following genes: the mutator gene *MLH1*, the unknown tumor suppressor gene at 8p22 and the metastatic gene *NM23-H1* and a positive correlation between LOH frequency in the *NM23-H1* gene and lymph node metastases.

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