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# Loss of heterozygosity for Rb locus and pRb immunostaining in laryngeal cancer: a clinicopathologic, molecular and immunohistochemical study

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**Abstract:** Several risk factors for the development of laryngeal cancer have been identified, such as smoking and alcohol consumption, but the molecular mechanisms related to the carcinogenesis in the larynx remain under investigation. In this context, deregulations of the cell-cycle-controling mechanisms, Rb-pathway in particular, have been suggested to be involved in the pathogenesis of laryngeal carcinoma. Our purpose was to investigate 13q14 LOH and the expression of Rb protein and their possible prognostic value in laryngeal cancer. The group of 67 patients with laryngeal cancer, surgically treated with minimum 5 years observation, was multi-variously analysed. LOH for Rb was investigated by PCR-based techniques using two microsatellite markers, D13S263 and D13S126, flanking the Rb locus. Amplification products from each polymorphism were fractionated by denaturing gel electrophoresis and detected by audioradiography. Immunohistochemical staining of paraffin specimens of laryngeal cancers was supervised by the use of monoclonal mouse antibodies IgG1 (Anti-Human Retinoblastoma Gene Product of Dako) in dilution of 1:50. Inactivation of Rb protein was assumed to represent the expression in ≤10 % tumour cells. The results of each examined individual factor were compared with clinicopathologic features and the results were statistically transformed (Chi-square test with Yates' correction, Mann-Whitney test). The Kaplan and Meier model was used for overall and disease free survival curves. Only p value of less than 0,05 was considered significant. 13q14 LOH was detected in 7/67 (10,4%) of informative tumours. No correlations were found between Rb genetic alteration (LOH) and gender, age, TNM staging, histological differentiation, nodal and local recurrences ($p>0.05$). There was a strong association between the loss of Rb and supraglottic localisation of tumour in the larynx ($p<0.01$). By univariate analysis 13q14 LOH proved to be significantly related to the overall survival whereas it was not related to the quicker relapse ($p=0.01$, $p>0.05$ respectively). The genetic data were correlated with the expression of the Rb protein ($p=0.001$). All tumours with Rb-LOH were immunohistochemically Rb-negative. Inactivation of Rb protein was observed in 9/67 cases (13.49%) and was significantly correlated with the polymorphism of cancer cells, but not with the histological grading. We also found the correlation between reduction of Rb protein and the size of primary tumour (T) ($p=0.03$) and local recurrence ($p=0.035$). There was no significant dependence between the level of Rb protein and other histopathological and clinical features ($p>0.05$). To conclude, analysis of 13q14 LOH enables the assessment of biology of laryngeal cancer and it can be a prognostic factor in overall survival. Immunohistochemical analysis of Rb protein expression in neoplastic cells made it easier to evaluate the mechanisms of cancerogenesis in laryngeal cancer and is closely related to genetic alteration in Rb locus.

**Key words:** laryngeal cancer, LOH for Rb, pRb, immunohistochemistry, prognostic factor

## Introduction

By compromising the normal mechanisms of speech, laryngeal cancer may cause a considerable morbidity despite its obvious effects on health and survival. Despite the high occurrence in men in Poland, relatively little is known about its molecular biology. There is a need for a deeper understanding of this disease as the 5-year survival rate for head and neck patients has not changed significantly over more than 20 years [1]. Although several risk factors for the development of laryngeal cancer have been identified, such as smoking and alcohol consumption, the molec-

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ular mechanisms related to the carcinogenesis in the larynx remain under investigation. In this context, deregulations of the cell-cycle-controlling mechanisms, Rb-pathway in particular, have been suggested to be involved in the pathogenesis of laryngeal carcinoma.

Carcinogenesis is a multistep process, which leads to the accumulation of several genetic alterations, including the inhibition of tumour suppressor genes (TSGs) and activation of oncogenes. The loss of inactivation of TSGs plays an important role in the development and progression of many solid tumours. There are more than 20 types of TSGs which are associated with human cancer. The inactivation of some tumour suppressor genes manifest itself through the loss of heterozygosity at nearby mapping markers. Studies on the loss of heterozygosity (LOH) have been carried out to identify sites harbouring tumour suppressor genes involved in tumour initiation or progression. Inactivation of these genes appears to have diagnostic and prognostic significance in some types of tumours. Molecular genetic and immunohistochemical tools based on suppressor inactivation might be very helpful in the treatment planning [2]. Gene products involved in the process of tumorigenesis and metastasis are expected to play a role in proliferation, migration and cell adhesion, and many such genes have been reported to be genetically altered in human invasive tumours. Some gene products are involved in proliferation (Rb, cyclin and cyclin-dependent kinases), cell-cell regulation, such as p53 and cyclin D1, and are predominantly associated with early stages of carcinogenesis. Others, such as E-cadherin and others adhesion molecules, are involved in cell-cell or cell-matrix adhesion, which are likely to affects invasive growth and metastasis [2-4].

The RB gene, located on chromosome 13q14, encodes a 110-kd key nuclear phosphoprotein implicated in the regulation of the transcription control mechanisms mediating progression throughout G1 phase of the cell cycle [3,5]. There are different mechanisms like homozygous deletion, promoter hypermethylation or point mutations within the coding sequence, which leads to the inactivation of pRB by releasing the pRB-bound E2F-members and transcription of the S-phase genes, promoting malignant transformation [4,6]. Abnormal pRB expression has been reported in different malignant tumours, such as lung, bladder, prostate and esophagus carcinomas and decreased pRB immunohistochemical nuclear staining indicating loss of Rb gene function has been reported in a high proportion of cases [7-10]. Genetic alterations involving the 13q14 region are common in human cancers although their role in the laryngeal cancer progression is still unclear.

Our purpose was to investigate 13q14 LOH and the expression of Rb protein and their possible prognostic value in laryngeal cancer.

**Table 1.** Characteristic of investigated group of patients with laryngeal cancer (N=67).

| Feature | | No. of patients | % |
|---|---|---|---|
| Gender | females | 9 | 13.4 |
| | males | 58 | 86.6 |
| Localisation of tumour in larynx | 1. epiglottic region | 31 | 46.3 |
| | 2. glottic | 35 | 52.2 |
| | 3. subglottic | 1 | 1.5 |
| Tumour size | T1 | 10 | 14.9 |
| | T2 | 5 | 7.5 |
| | T3 | 16 | 23.9 |
| | T4 | 36 | 53.7 |
| Nodal metastases | N0 | 42 | 62.7 |
| | N1 | 5 | 7.5 |
| | N2a | 4 | 6.0 |
| | N2b | 13 | 19.4 |
| | N2c | 3 | 4,4 |
| Clinical staging | TNM I | 9 | 13.4 |
| | TNM II | 9 | 13.4 |
| | TNM III | 21 | 31.3 |
| | TNM IV | 28 | 41,9 |
| Local recurrences | present | 4 | 6.0 |
| | absent | 39 | 94.0 |
| Nodal recurrences | present | 12 | 17.9 |
| | absent | 55 | 82.1 |
| Histological grading | G1 | 6 | 8.9 |
| | G2 | 43 | 64.2 |
| | G3 | 18 | 26.9 |

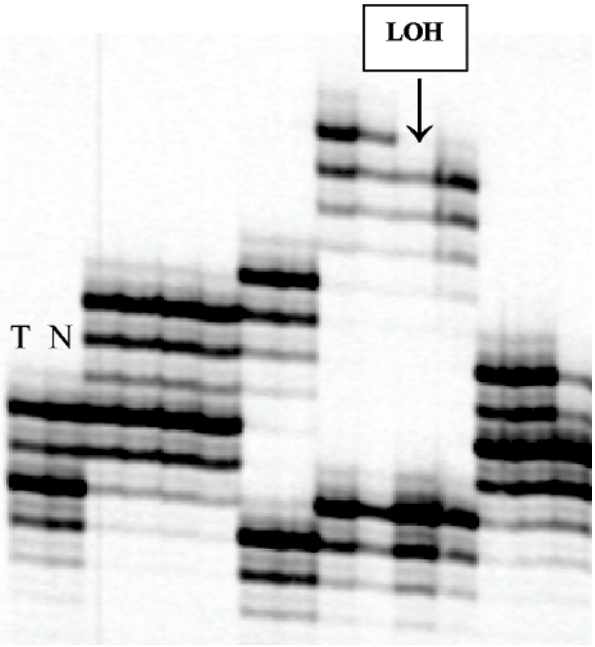


**Fig. 1.** Representative autoradiographs from loss of heterozygosity analysis of chromosome 13q14 in laryngeal cancer. DNAs extracted from tumour (T) and corresponding normal (N) tissues were analysed using microsatellite marker D13S153.


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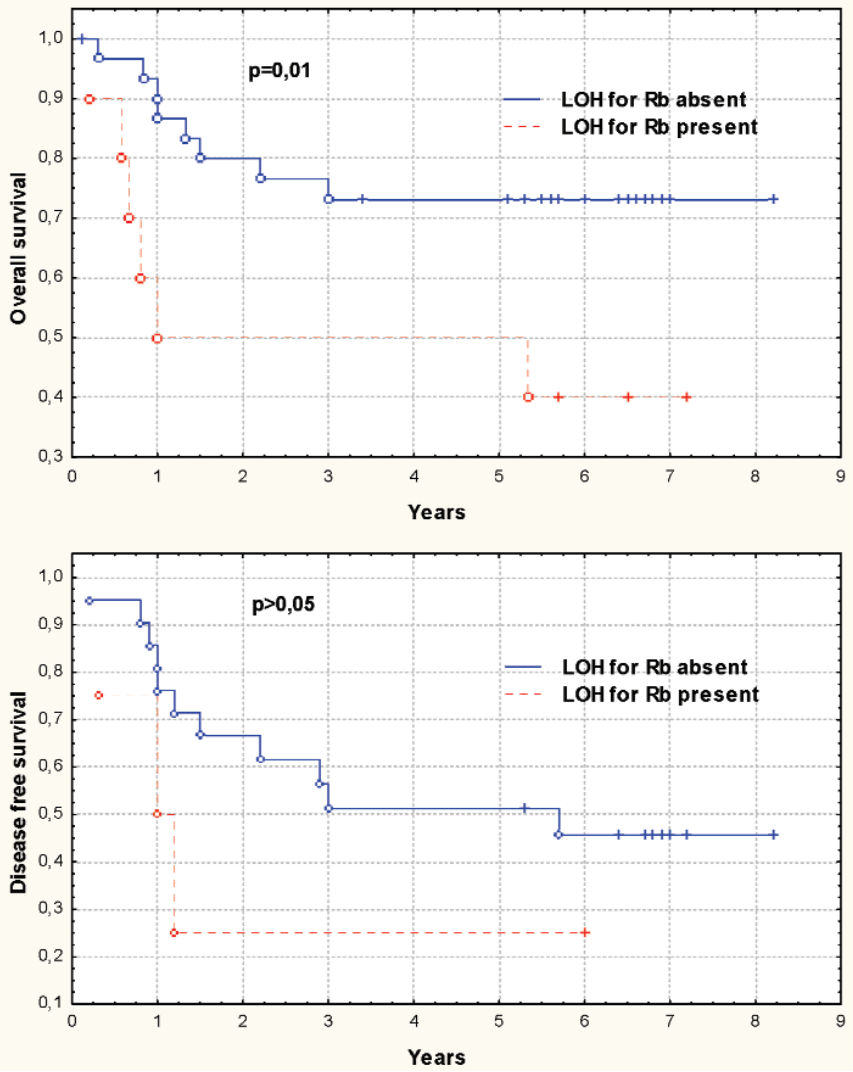


**Fig. 2.** Overall and disease free survival in patients with laryngeal cancer according to LOH for Rb.

## Material and methods

**Patients.** The group of 67 patients with laryngeal cancer, surgically treated with minimum 5 years observation, was studies using multivariate analysis (Table 1).

**LOH analysis**. The occurrence of homozygous deletions was investigated by PCR. Only one tumour DNA samples showed reduction (50%) in the intensity of the bands in the autoradiograms when compared with the corresponding normal DNA, suggesting the occurrence of homozygous deletion. LOH was analysed by PCR-based techniques using two microsatellite markers, D13S263 and D13S126, flanking the Rb locus. Amplification products from each polymorphism were fractionated by denaturing gel electrophoresis, and were detected by audioradiography.

**Immunohistochemistry**. The archival paraffin embedded tissue sections 5 µm thick were stained with hematoxilin and eosin (H+E) for routine morphological examination. Immunohistochemical staining of paraffin specimens was supervised by the use of monoclonal mouse antibodies IgG1 (Anti-Human Retinoblastoma Gene Product of Dako) in dilution of 1:50. In order to expose the antigen, sections were placed in the warmed citric buffer (pH=6.0) and afterwards they were put into a microwave of 630 W for 18 minutes. The incubation time with the antibody was 30 minutes. For the detection LSAB HRP+kit and DAB (3'3'diaminobenzidine) as chromogen were used to manifest immunohistochemical reactions. The morphometric evaluation of examined specimens was performed in the light microscope (Nikon, magnification ×200 and ×400) coupled with a computer equipped with a morphometric program. Rb protein in laryngeal cancer was quantified by calculating the index representing the ratio of cells with the positive reaction to the entire number of the assessed tumour cells in examined area. Inactivation of Rb protein was assumed to represent the expression in ≤10% tumour cells.

**Statistical analysis**. The LOH for Rb and Rb protein expression was estimated and statistical data were processed by the computer program STATISTICA PL 6.0. The results of each individual factor examined were compared with age, gender, clinical staging (TNM), histological grading, local and nodal recurrences, and overall and disease free survival of the patients. The results were statistically transformed (Chi-square test with Yates' correction, Mann-Whitney test). The Kaplan and Meier model was used for overall and disease free survival curves. Only p value of less than 0.05 were considered significant.

## Results

13q14 LOH was detected in 7/67 (10.4%) of informative tumours (Fig. 1). In the series of examined


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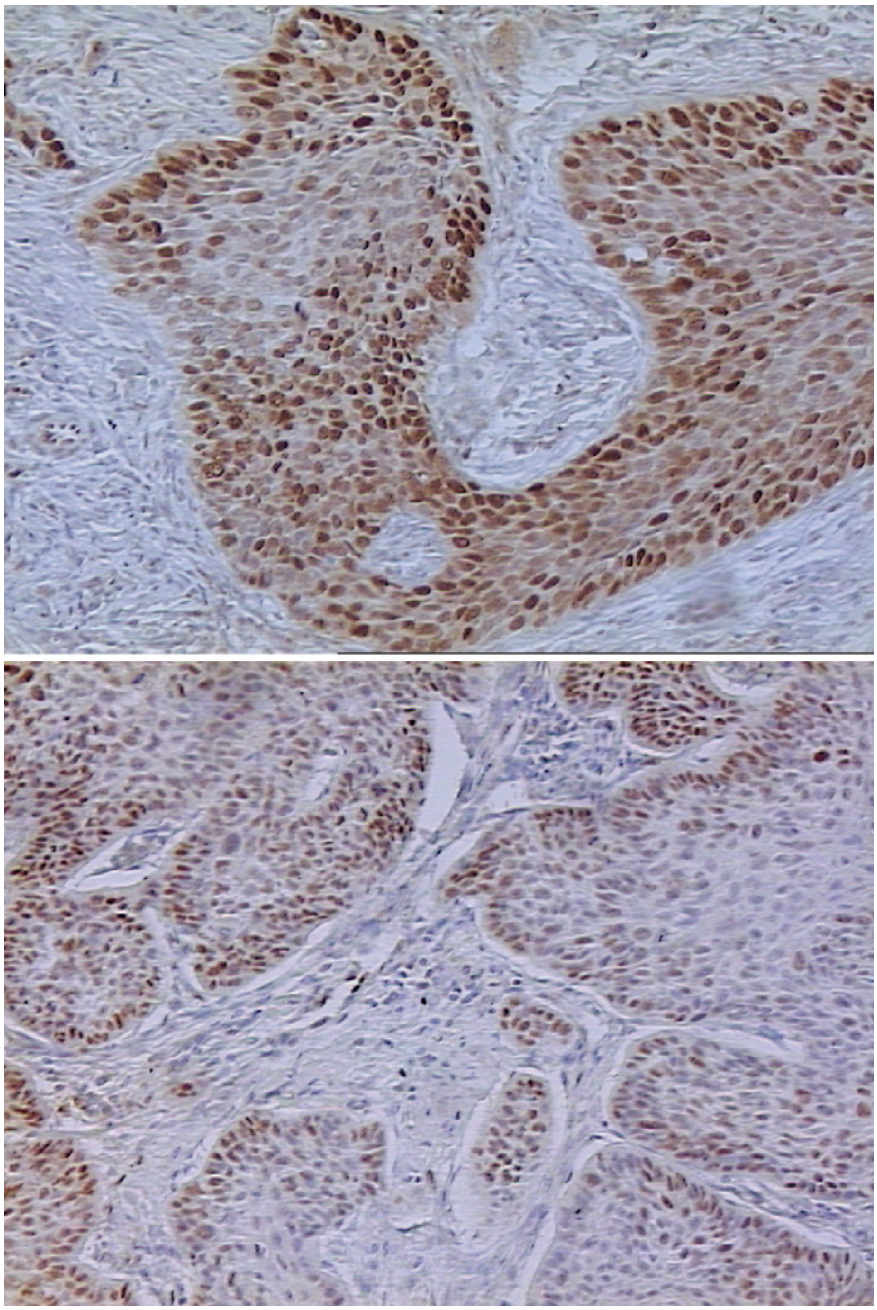

**Fig. 3**. Expression of Rb protein – strong brownish nuclear immunohistochemical staining in laryngeal neoplastic cells, especially seen on tumour front (immunoperoxidase reaction, magnification ×100).

tumours no correlation was found between Rb genetic alteration (LOH) and gender, TNM staging, histological differentiation, nodal and local recurrences ($p>0.05$). There was a strong correlation between loss of Rb and supraglottic localisation of tumour in the larynx ($p<0.01$).

LOH for Rb positivity was associated with a younger age at diagnosis (<45 y.o.), but it was not significant. By univariate analysis 13q14 LOH proved to be significantly related to overall survival whereas it was not related to quicker relapse ($p=0.01$, $p>0.05$ respectively) (Fig. 2).

The genetic data were correlated with the expression of the Rb protein examined by immunohistochemistry ($p=0.001$). All tumours with Rb-LOH were immunohistochemically Rb-negative.

The patterns of Rb protein expression was exclusively nuclear in range between 0-85% with the strongest intensity in proliferative tumour front (Fig. 3).

Inactivation of Rb protein was observed in 9/67 cases (13.49%) and was significantly correlated with polymorphism of cancer cells. More advanced polymorphism and differentiation of cancer cells were related to higher Rb protein expression. We did not find any significant correlation between Rb expression and histological grading although in low differentiated tumours (G3) the reduction in this protein expression (<10% stained cancer cells) was seen more often. We


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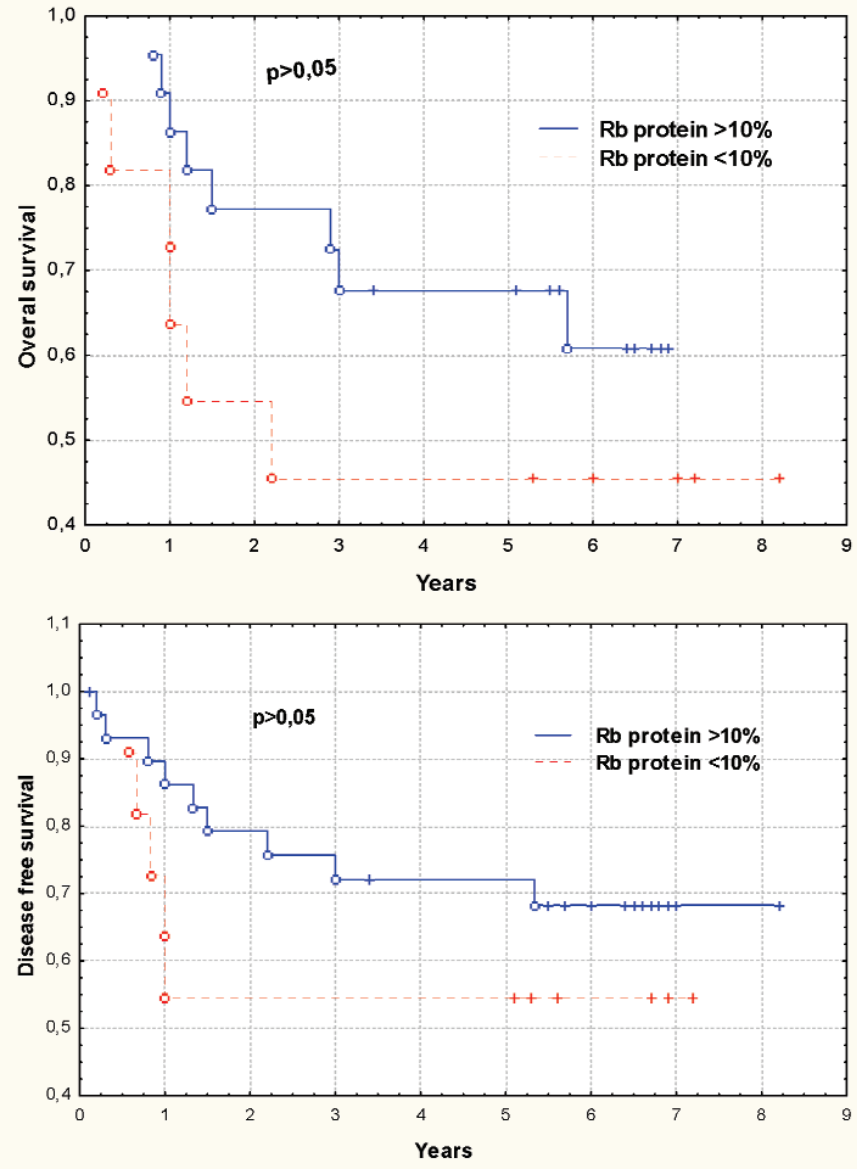


**Fig. 4**. Overall and disease free survival in patients with laryngeal cancer according to Rb protein.

also found correlation between the size of primary tumour (T feature) and Rb protein expression (p=0.03), which was absent or low in advanced laryngeal cancers (<10%). It was noticed, that Rb expression was reduced in cases of local recurrence (p=0.035). We found the inactivation of Rb protein more often in cases with low histological grading (G3) but correlations were not significant (p>0.05). There was no significant dependence between the level of Rb protein and gender, age of the patients, localization of tumour in larynx, nodal and distant metastases (N and M features), nodal recurrence or overall and disease free survival of the patients (p>0.05) (Fig. 4).

## Discussion

This study analysed the allelic status of the Rb gene and its protein product expression in different stages of laryngeal cancer. Consistent with previous observation in head and neck cancer, it was found that 7 (10.4%) of the 67 informative cases had LOH of the Rb gene [11-13]. In our study low frequency of loss for Rb confirms the results obtained by Lee in head and neck cancer (15%) and Kannan in oral cancer (17%) [11,13]. These data are not consistent with the significant and high frequency of allelic loss reported for the same Rb marker in laryngeal cancer by Scholnick *et al.* [1]. Loss of heterozygosity at the retinoblastoma gene in the mentioned study of laryngeal supraglottic cancer [1] occurred at a frequency (59%) close to that found in non small cell cancer (48%) [14] or oesophageal cancer (54%) [10]. Nevertheless, for head and neck cancer LOH for Rb is rather infrequent [11-13]. Although, the reason for this high percentage of LOH for Rb in those studies might be connected with the chosen investigated group related only to supraglottic


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laryngeal cancer cases. There is a well known a genuine biologic difference between supraglottic and glottic patients. Glottis and supraglottis, although anatomically interconnected, are embryologically distinct [15]. Moreover, squamous cell carcinomas originated from these subsites, differ in terms of epidemiology, risk factors, clinical behavior and prognosis. The patients with supraglottic cancer usually have a poorer prognosis, as well as more advanced (usually with nodal metastases in the neck) and histologically aggressive tumours than the patients with glottic tumours [15]. In our analysis there was a strong correlation between allelic loss of Rb and supraglottic localisation of tumour in the larynx (p=0.001).

Changes in the expression of Rb gene, which is localized in 13q14, correlate with the presence of Rb mutations and they occur relatively rarely in sqamous cell carcinoma (SCC), but it is believed that they are an early event. It was proved that Rb gene mutation plays a role in the mechanism of metastasis to the local lymph nodes in the laryngeal cancer but our study did not confirm that [16-18]. We did not find any significant correlation between Rb LOH and clinical stage, histological grade, local and nodal recurrences. Nevertheless, Rb LOH was demonstrated at early (2/67, I-II stages) and advanced (5/67, III-IV stages) clinical stages of tumour, suggesting that LOH at the Rb locus occurs before the clonal expansion of the tumour. Allelic imbalance of the Rb gene in our study was associated with the decrease in pRb protein expression as well. The connection between Rb LOH and altered pRb expression was significant ($p<0.01$). Association between LOH on 13q where Rb locus is located, and the decreased pRb expression was observed in many tumours, such as lung, liver, bladder, oesophagus, endometrial cancer and chondrosarcoma [10,19-23]. Our data suggest that LOH at Rb locus plays a role in the tumorigenesis of a subset of laryngeal cancers and correspond with the altered expression of the pRb. In this study the inactivation of Rb protein and 13q14 LOH were detected in almost the same percentage of cases (10.4% and 13.4% respectively). These observations indicate that LOH for Rb presents the major mechanism which leads to pRb inactivation. Moreover it was found in our study that LOH of chromosomal region 13q14 has a prognostic value in the assessment of overall survival of patients surgically treated for laryngeal cancer. This might suggest that Rb genetic alteration affects not only the early development of laryngeal cancer but also further tumour progression.

In the presented data, the evidence of Rb gene and protein inactivation was found in a low proportion of laryngeal tumours examined and it may indicates Rb as a minor target of inactivation in laryngeal cancer. Although Volavsek *et al.* with their results indicated that the loss of Rb expression in SCC cases showed a significant association with tumour grade and that low frequency of Rb gene inactivation might be more important in the development of SCC than it was previously considered. They also highlighted the central role of cyclin D1 in the regulation of the cell cycle in head and neck cancer [24]. Cyclin D1 overexpression (detected by IHC methods) has been described as being able to supersede Rb-mediated growth inhibition in SCC. The authors claim that a significant consistency between immunohistochemical and molecular investigations of cyclin D1 and Rb indicate that pRb immunostaining can be used as a reliable marker of the Rb gene status [24].

The clinical relevance of pRb expression in human tumours is still controversial: low levels of this protein have been found to correlate with a worse prognosis in prostate, breast, endometrial, head and neck cancer but not in bladder and gastric cancers [10,19,20,25-28]. In these data the loss of pRb protein was observed in 9/67 cases (13.4) and was significantly correlated with T status (p=0.03) and local recurrence (p=0.035). Those parameters are considered to be the predictors of poor prognosis in laryngeal cancer. Although, to make Rb protein expression level more reliable prognostic factor in head and neck carcinoma, further investigation should be made perhaps involving detection of its phosphorylation status. Although, all data, to our knowledge, suggest that loss of pRb may contribute to the multistage of head and neck carcinogenesis.

To conclude, analysis of 13q14 LOH enables the assessment of biology of laryngeal cancer, which can be a prognostic factor of overall survival. Although the identity of other critical genetic loci should be revealed by the evaluation of other chromosomal arms. Immunohistochemical analysis of Rb protein expression in neoplastic cells made it easier to evaluate the mechanisms of cancerogenesis in laryngeal cancer and is closely related to genetic alteration in Rb locus.

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