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# Deletions of *N33*, *STK11* and *TP53* are involved in the development of lymph node metastasis in larynx and pharynx carcinomas

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**Abstract.** *Background:* Lymph node metastasis is the mayor cause of mortality in patients with head and neck squamous cell carcinomas (45%). The genetic changes underlying metastasis are still largely unknown and genetic markers to predict lymph node positivity still need to be found. The aim of this study was to search such markers by using Multiplex Ligation-dependent Probe Amplification (MLPA), a semi-quantitative PCR technique to detect gene copy number alterations. *Methods:* Thirty-seven genes were analysed by MLPA in 34 larynx and 22 pharynx carcinomas. *Results:* Losses of *CDKN2A* (9p21) and *MLH1* (3p22) and gains of *CCND1*, *EMS1* (both at 11q13), *RECQL4* and *PTP4A3* (both at 8q24) were the most frequent aberrations in both larynx and pharynx carcinomas. Amplifications were detected at *EMS1, CCND1* and *ERBB2* (17q21). A correlation between loss of *N33* (8p22) and poor survival was found ($p = 0.02$). Gain of *EMS1* had the same relation with survival but not significant ($p = 0.08$). Lymph node positive tumors presented a specific pattern of genetic alterations, with losses of *N33*, *STK11* (19p13) and *TP53* (17p13), the latter especially in larynx tumors. *Conclusion:* We propose that these 3 genes might play a role in the development of metastasis in larynx and pharynx squamous cell carcinomas.

Keywords: Larynx and pharynx carcinoma, MLPA, lymph node metastasis, genetic alterations

## 1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent male cancer in the western world. In the last decades, important advances in diagnosis tools and treatment have been made [15]. However, tumors are still diagnosed in late stages and survival rates have hardly improved. Although tumors with the same TNM classification can have a variable evolution, lymph node status is the most important factor associated to survival [23].

HNSCC are known to develop and accumulate a very complex chromosomal alterations. Many genetic studies have been applied using different cytogenetic techniques, such as loss of heterozygosity (LOH), fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), array-CGH and spectral karyotyping (SKY) [8–10,32,34]. A number of chromosomal regions are consistently altered in these tumors, including losses of 3p and 9p and gains of 3q, 5p, 8q and 11q13. In addition a number of recurring amplifications have been found at 3q24-qter, 11q13, 18p, 18q11.2, 8q23-24 and 11q14-22 [4,9,33]. Many important genes related with proliferation and cell cycle regulation have been identified in these chromosomal regions: *CDKN2D* at 9p21 encodes the cell cycle inhibitor p16; *CCND1* and *EMS1* at 11q13 are associated with cell cycle regulation; *c-MYC, PTP4A3, RECQL4* at 8q23-24 are related to transcription regulation and apoptotic functions; *hTERT* at 5p15 with telomerase activity; and *MLH1* at 3p22 with DNA repair function.

A tumorigenesis model based on LOH studies has been postulated in HNSCC and proposed loss of 9p21

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as an early event, followed by losses of 3p21 and 17p13 and continued by losses of 11q13, 13q21, 14q31, 6p, 8p, 4q26 [5]. But there is no information on the role of frequent gains and amplifications which should be important as well in the development of HNSCC. Moreover, this model does not include the steps towards lymph node metastases. Other reports have addressed this topic and studied chromosomal aberrations [4,35, 38], allelic losses [34] and mRNA and protein expression patterns [6,26] in relation to metastasis development. However, the majority of the data generated were not in agreement. Loss of 8p and 9p and gain of 3q have been frequently described but only amplifications of chromosome band 11q13 and overexpression of genes in this region have repeatedly been implicated in metastasis. Even so, 11q13 is a region densely packed with genes and it is still not clear whether *CCND1*, *EMS1* or perhaps another gene in the amplicon is the true responsible gene involved in metastasis.

In this paper, we studied gene copy number changes by Multiplex Ligation-dependent Probe Amplification (MLPA), a rapid, semiquantitative and high resolution PCR technique, capable of analysing gene copy number alterations of up to 40 genes in a single experiment, with the use of just one pair of PCR primers [28]. The targets sequences were chosen in genes or chromosomal regions that have been described in the literature to be often altered in different types of solid tumors.

We studied a series of 56 primary larynx and pharynx squamous cell carcinomas by MLPA aiming to find genetic alterations in relation with lymph node involvement.

## 2. Material and method

We studied 56 primary tumors (34 larynx and 22 pharynx squamous cell carcinomas) from male patients with larynx and pharynx squamous cell carcinomas diagnosed between 1993 and 1999. The mean age was 63 years (range 44–80) and all had a custom consumption of tobacco and alcohol. Patients underwent surgery (i.e. laryngectomy or pharyngectomy with neck dissection). Resection margins were free of tumor in all cases. Informed consent was obtained from all patients and the study was approved by the Hospital Valle del Nalón, Asturias, Spain. Twenty-one patients (37%) received radiotherapy after surgery. Thirty patients were lymph node negative (54%) and 26 lymph node positive (46%). Clinico-pathological and cytometric characteristics were collected and follow-up information was available of at least 5 years or until the death of the patient (range 0–100 months). A summary of all data is showed in Table 1.

Tissue samples of the primary tumors were obtained from surgical resection specimens of non-necrotic tumor areas and stored in liquid nitrogen.

Tumor DNA was extracted using phenol-chloroform extraction following a standard DNA isolation protocol. Normal reference DNA was obtained from blood of healthy donors using the Qiagen DNA isolation kit, according to the manufacturer's recommendations (Qiagen GmbH, Hilden, Germany).

The MLPA technique has been performed as described in detail previously [28]. A MLPA "SALSA P084 HNSCC1" kit was obtained from MRC-Holland, Amsterdam, the Netherlands. This probe mixture contained 42 probes for specific genes located in chromosomal regions that have been described in the literature to be often altered in HNSCC (*www.mlpa.com*). Each probe is composed of two parts that hybridize to adjacent target sequences in the DNA. After a ligation step and a PCR amplification, each probe gives rise to a product with a unique size between 130 and 480 bp. Briefly, 100 ng DNA was denatured at 98°C for 5 min and hybridized with the MLPA probe mixture at 60°C for 16 hours. Ligation of the two parts of each probe was preformed by a thermostable ligase. All probe ligation products have the same end sequences and were amplified by PCR using the same primer pair for 60°C 1 min, 33 cycles of 95°C 30 s, 60°C 30 s and 72°C 1 min, followed by 20 min at 72°C and kept cold at 4°C. The products were subsequently analysed on an ABI Prism 3100 sequencer and by GeneScan v3.7 software (Applied Biosystems, Warrington, UK).

From the 42 MLPA probes used in this study, 5 probes did not work correctly in our hands and were discarded of the analysis. Fourteen control experiments using normal DNA from 7 different donors were used to calculate median reference values and the standard deviations for every probe. Each tumor sample was analysed at least twice. For every gene, the relative copy number was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. We observed that a MLPA experiment analysing a new normal DNA resulted in relative copy numbers varying between 0.85 and 1.15, including the standard deviation, for each probe. Therefore we decided that relative copy number values, including the standard deviation, lower than 0.8 were interpreted as losses, higher than 1.2 as gains and 2.5 or higher as amplifications.

Table 1
Summary of the clinico-pathological and cytometric characteristics

| | Clinico-pathological characteristics | | | | | | |
|---|---|---|---|---|---|---|---|
| | Larynx ($n = 34$) | | | Pharynx ($n = 22$) | | | Total (%) ($n = 56$) |
| | Supraglottis | Glottis* | Total (%) | Oropharynx | Hypopharynx | Total (%) | |
| Age (years) | 65 | 61 | 63 | 58 | 62 | 60 | 62 |
| (range) | (48–77) | (43–80) | (43–80) | (44–72) | (50–74) | (44–74) | (43–80) |
| Localization | 19 | 15 | 34 | 11 | 11 | 22 | 56 |
| Stage | | | | | | | |
| I | 5 | 3 | 8 (24) | 1 | 0 | 1 (5) | 9 (16) |
| II | 7 | 4 | 11 (32) | 1 | 1 | 2 (9) | 13 (23) |
| III | 1 | 3 | 4 (12) | 3 | 2 | 5 (23) | 9 (16) |
| IV | 6 | 5 | 11 (32) | 6 | 8 | 14 (64) | 25 (45) |
| Histological grade | | | | | | | |
| Poor | 3 | 2 | 5 (15) | 4 | 2 | 6 (27) | 11 (20) |
| Moderate | 11 | 7 | 18 (53) | 5 | 5 | 10 (46) | 28 (50) |
| Good | 5 | 6 | 11 (32) | 2 | 4 | 6 (27) | 17 (30) |
| Lymph node | | | | | | | |
| Negative ($N_0$) | 12 | 10 | 22 (65) | 4 | 4 | 8 (36) | 30 (54) |
| Positive ($N_+$) | 7 | 5 | 12 (35) | 7 | 7 | 14 (64) | 26 (46) |
| Postoperative-radiotherapy | | | | | | | |
| No | 14 | 8 | 22 (65) | 6 | 7 | 13 (59) | 35 (63) |
| Yes | 5 | 7 | 12 (35) | 5 | 4 | 9 (41) | 21 (38) |
| Status disease | | | | | | | |
| Without tumor | 13 | 7 | 20 (59) | 3 | 2 | 5 (23) | 25 (45) |
| Recurrence | 3 | 4 | 7 (21) | 4 | 7 | 11 (50) | 18 (32) |
| Distant metastasis | 2 | 1 | 3 (9) | 1 | 2 | 3 (14) | 6 (11) |
| Second primary tumor | 1 | 3 | 4 (12) | 3 | 0 | 3 (14) | 7 (13)** |
| Status patient | | | | | | | |
| Alive | 8 | 6 | 14 (41) | 1 | 2 | 3 (14) | 17 (30) |
| 👽 Recurrence | 3 | 4 | 7 (21) | 4 | 7 | 11 (50) | 18 (32) |
| 👽 Distant metastasis | 2 | 1 | 3 (9) | 1 | 2 | 3 (14) | 6 (11) |
| 👽 Second primary tumor | 1 | 3 | 4 (12) | 3 | 0 | 3 (14) | 7 (13) |
| 👽 Other diseases | 3 | 1 | 4 (12) | 0 | 0 | 0 (0) | 4 (7) |
| 👽 Postoperative | 2 | 0 | 2 (6) | 2 | 0 | 2 (9) | 4 (7) |
| | Cytometric characteristics | | | | | | |
| | Larynx ($n = 28$) | | | Pharynx ($n = 20$) | | | Total (%) ($n = 48$) |
| | Supraglottis | Glottis* | Total (%) | Oropharynx | Hypopharynx | Total (%) | |
| DNA ploidy | | | | | | | |
| Diploid | 6 | 7 | 13 (46) | 3 | 2 | 5 (25) | 18 (37) |
| Aneuploid | 10 | 5 | 15 (54) | 6 | 9 | 15 (75) | 30 (63) |
| S-Phase$^{\Sigma}$ | | | | | | | |
| Low | 9 | 6 | 15 (54) | 8 | 7 | 15 (75) | 30 (63) |
| High | 7 | 6 | 13 (46) | 1 | 4 | 5 (25) | 18 (37) |

*Include glottis, transglottis and subglottis. **Second primary tumor: 3 lung, 1 trachea, 1 hypopharynx, 1 oropharynx and 1 colon.

👽: died by.

$^{\Sigma}$Low S-phase = %S-phase <10% in diploid and <20% in aneuploid. High S-phase = %S-phase >10% in diploid and >20% in aneuploid [9].

The statistic analysis was carried out using Student's test, Pearson and Fisher Chi-square test. Survival estimation was analysed by Kaplan–Meier statistics with the log rank test. $p$-values $< 0.05$ were considered significant. Losses, gains and amplifications were tested for correlation with the clinico-pathological and cytometric characteristics (age, localization, stage, histological grade, lymph node status, postoperative radiotherapy, disease-free survival, ploidy and S-phase).

## 3. Results

Thirty-seven out of the 42 probes in the MLPA kit worked reproducibly in our hands. Gene copy number alterations were found in all 56 cases. All gains and losses detected by MLPA are shown in Fig. 1. Losses of *CDKN2A* at 9p21 (42 cases, 75%) and *MLH1* at 3p21 (36 cases, 64.3%) and gains of *CCND1* (31 cases, 55.4%) and *EMS1* (30 cases, 53.6%) at 11q13 were the most recurrent alterations in both groups. Other recurrent changes involved losses of *CASP6* at 4q25 (29 cases, 51.8%) and *IGSF4* (28 cases, 50%) and *IL18* (26 cases, 46.4%) both at 11q23, and gains of *RECQL4* (27 cases, 48.2%) and *PTP4A3* (24 cases, 42.9%), both at 8q24.3.

Fifty-four amplifications were detected. Genes amplified in more than two cases were *CCND1* at 11q13 (21 cases, 37.5%), *EMS1* at 11q13 (19 cases, 33.9%), *PTP4A3* at 8q24.3 and *ERBB2* at 17q21 (3 cases, 5.4%). In Table 2 the distribution of all amplifications is presented in relation with the lymph node status of the tumor.

$N_+$ tumors (primary tumors from patients with positive lymph nodes) showed significantly more losses of *TP53*, *N33* and *STK11* than $N_0$ tumors (primary tumors from patients without positive lymph nodes) (Fig. 2). Gains of *EMS1*, *CCND1* and *PTP4A3* were also found more frequently in $N_+$ tumors but none of these differences were statistically significant (Fig. 2). Loss of *TP53* was also related to laryngeal $N_+$ tumors ($0/22$ $N_0$ tumors *versus* $6/12$ $N_+$ tumors; Fisher exact,

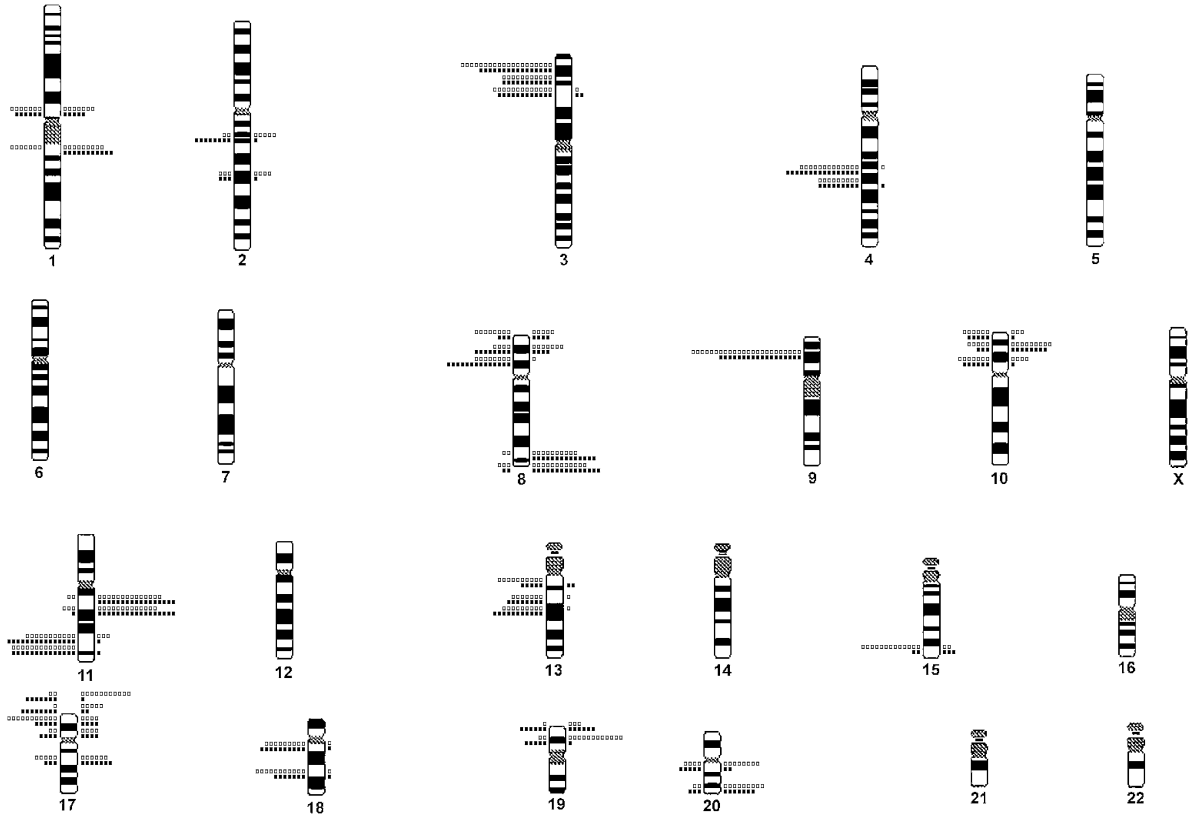


Fig. 1. Genetic alterations in 56 larynx and pharynx primary carcinomas detected by MLPA. Squares to the right of each chromosome represent gains and squares to the left represent losses. White squares represent lymph node negative tumors and black squares represent lymph node positive tumors.

Table 2
Amplifications detected by MLPA in 34 larynx and 22 pharynx tumors

| Amplification | Larynx ($n = 34$) | | Pharynx ($n = 22$) | |
|---|---|---|---|---|
| | 22 $N_0$ tumors | 12 $N_+$ tumors | 8 $N_0$ tumors | 14 $N_+$ tumors |
| *CCND1* | 6 cases, 27% | 5 cases, 42% | 5 cases, 63% | 5 cases, 36% |
| *EMS1* | 3 cases, 14% | 5 cases, 42% | 5 cases, 63% | 6 cases, 43% |
| *PTP4A3* | 1 case, 5% | 1 case, 8% | 0 case, 0% | 1 case, 7% |
| *ERBB2* | 0 cases, 0% | 2 cases, 17% | 0 case, 0% | 1 case, 7% |
| *LMNA* | 1 case, 5% | 0 cases, 0% | 0 case, 0% | 1 case, 7% |
| *MFHAS1* | 1 case, 5% | 0 cases, 0% | 0 case, 0% | 1 case, 7% |
| *RECQL4* | 1 case, 5% | 0 cases, 0% | 0 case, 0% | 1 case, 7% |
| *CTSB* | 0 cases, 0% | 1 case, 8% | 0 case, 0% | 0 cases, 0% |
| *TP53* | 0 cases, 0% | 0 cases, 0% | 0 case, 0% | 1 case, 7% |

$N_0$ tumors = primary tumors from patients without positive lymph nodes; $N_+$ tumors = primary tumors from patients with positive lymph nodes.

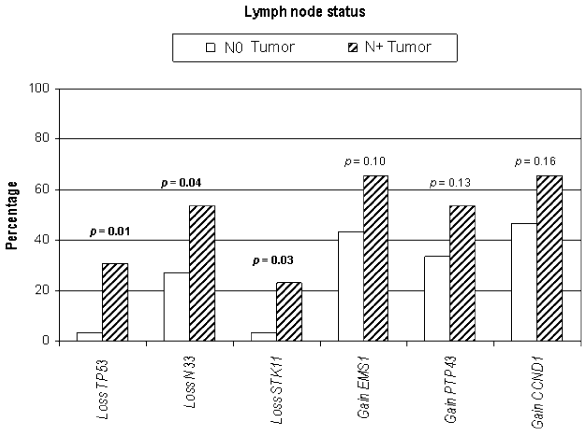


Fig. 2. Genetic alterations associated to lymph node positive larynx and pharynx tumors detected by MLPA.

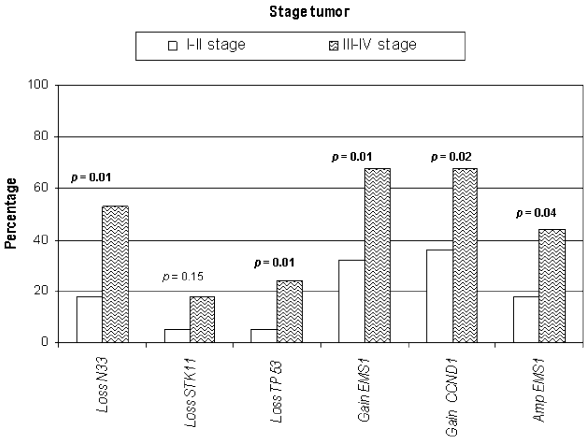


Fig. 3. Genetic alterations associated to advanced stage (III and IV stage) from larynx and pharynx tumors detected by MLPA. (Amp: amplification).

$p = 0.001$) but not to pharyngeal $N_+$ tumors ($1/8$ $N_0$ tumors *versus* $2/14$ $N_+$ tumors; Fisher exact, $p = 0.7$).

All amplifications at *ERBB2* (3 cases; Table 2) were found in $N_+$ tumors that were stage IV and DNA aneuploid. Amplification of *EMS1* was found in 42% of the $N_+$ tumors (11 cases out of 26) and in 26% of the $N_0$ tumors (8 cases out of 30).

Losses of *N33*, *TP53* and *STK11* and gains of *CCND1* and *EMS1* correlated with advanced stages (III and IV stage) (Fig. 3). Amplification of *EMS1* showed correlation to stage IV tumors (Fisher exact, $p = 0.04$; Fig. 3).

Survival was significantly worse in cases with loss of *N33* (Kaplan–Meier, $p = 0.02$; Fig. 4). Gain of *EMS1* had the same tendency but was not significant (Kaplan–Meier, $p = 0.08$).

## 4. Discussion

In a previous study, we analysed the present series of tumors by conventional CGH in an attempt to find chromosomal losses, gains and/or amplifications related to clinical outcome [11]. A very high number of aberrations were found both in $N_0$ as in $N_+$ tumors and only amplification of 11q13 (6 out of 56 cases) correlated with advanced tumor stage and lymph node positivity. However, we were unable to find significant correlations with survival. Therefore we undertook this new study using MLPA, a technique that provides copy number information of gene level.

In this study, the MLPA kit P084 was chosen for enabling the analysis of genes located in chromosomal regions that according to literature may play a role in HNSCC. We are aware of the possibility that the se-

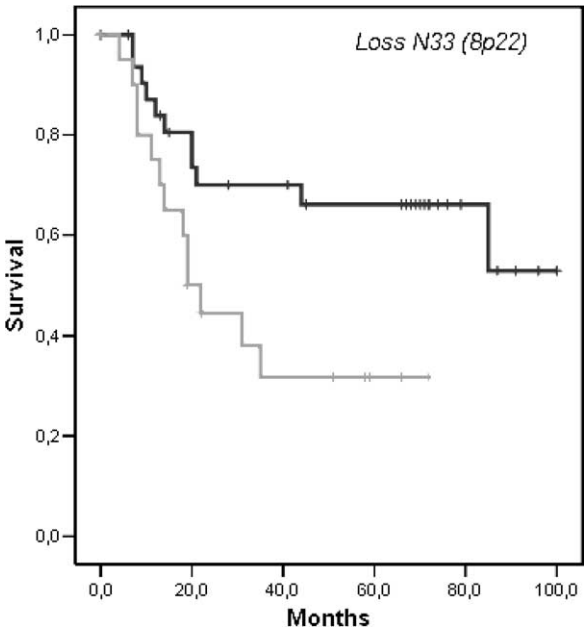


Fig. 4. Kaplan–Meier survival curve of loss of gene *N33* detected by MLPA in 56 larynx and pharynx squamous cell carcinomas ($p = 0.02$). The black line represents cases without loss of *N33* and the gray line cases with loss of *N33*.

lected genes in the kit may not be the ones that have triggered the occurrence of a certain gain or loss of the chromosomal region and that a neighbouring gene may well be more important. Thus frequent losses were found for genes located in chromosome arms 3p, 8p, 11qter and 18q, and gains at 8qter and 11q13 which is in agreement with previous chromosomal studies [4, 11,35,38]. Probably due to its higher sensitivity, MLPA detected higher frequencies of losses of genes located at 9p21 and gains/amplifications at 11q13. Unfortunately, MLPA kit P084 contained no probes for genes at 3q, 5p or 18p, regions that we know from literature and our own CGH study to frequently present copy number gains in HNSCC [11,19,21,31].

In the 56 larynx and pharynx carcinomas, the most frequent losses were of *CDKN2A* (9p21) and *MLH1* (3p22) (Fig. 1). This is not surprising, when considering that deletions in both chromosomal regions have been described as an early event in the HNSCC development [4,5,34]. In our series, neither of these two genes correlated to clinico-pathological data, and stage I and II tumors showed about the same frequencies as stage III and IV tumors.

The most frequent copy number gains were found of *CCND1* and *EMS1* (both in 11q13), as well as *RECQL4* and *PTP4A3* (both in 8q24). Both chromosomal regions are involved in gains and amplifications in many different tumors such as breast, lung, bladder and esophageal cancer, and several candidate genes have been suggested. In 8q24, *PTP4A3* (*PRL3*) was proposed in colorectal adenocarcinomas as an important gene involved in metastasis and also *FAK* as a regulator of cell invasion and promotor of motility in HNSCC [12,27]. In this study, neither *PTP4A3* nor *RECQL4* in 8q24 (*FAK* was not represented in the MLPA kit) correlated to clinical data. In 11q13, both *CCND1* and *EMS1* are claimed to play a role in tumor progression and metastasis, although other genes, e.g. *FADD*, *EMSY* or *TAOS1* may be equally important [13,14,25,29]. We found that gains of *EMS1* and *CCND1* related to lymph node positivity and significantly correlated to advanced tumor stage (Figs 2 and 3). Interestingly, *EMS1* gain but not *CCND1* related to poor survival but not significantly (Kaplan–Meier, $p = 0.08$). Moreover, amplification of EMS1 but not of *CCND1* was correlated to advanced stage of the tumor (Fisher exact, $p = 0.04$; Fig. 4). This confirms that amplification of *EMS1* occurs independently from *CCND1* amplification as has been found in others studies in HNSCC [17,25]. Besides, overexpression of cortactin, the cytoskeletal protein encoded by *EMS1* (also named *CTTN*) has been described to enhance cellular motility and might play a important role in tumoral invasion [16].

The most interesting findings of our study concerned copy number losses of 3 genes which, although less frequently observed, were found related to clinicopathological data: *N33*, *TP53* and *STK11*. All three losses associated to $N_+$ tumors and advanced stage (Figs 2 and 3) and *N33* also correlated to survival (Fig. 4). Larynx tumors present less frequently positive lymph nodes than pharynx tumors; in our series 12 out of 34 cases (36%) in larynx *versus* 14 out of 22 (64%) in pharynx were lymph node positive (Table 1). In a separate analysis of the 34 larynx and the 22 pharynx tumors we found that only loss of *TP53* were correlated with $N_+$ larynx tumors but not with $N_+$ pharynx tumors.

P53 protein is a key regulator of the cell cycle and apoptosis functions. Loss of p53 function has been detected in HNSCC and also in a many other types of tumor [1]. Braakhuis et al. proposed P53 mutation as one of the earliest events giving rise to a genetic altered area (field cancerization) with malignant potential in tumorigenesis [2]. Furthermore, p53 gene function alterations, together with *CCND1* overexpression in early phases of the tumoral progression, has been described to increase genetic instability and, con-

sequently, an accumulative number of new genetic and chromosomal alterations [1,36]. In our study, loss of *TP53* related to lymph node positivity and late phase progression step. Therefore, it may be speculated that its function in apoptosis control, rather than of guardian of genomic stability, is involved.

Inherited mutation of the *STK11/LKB1* tumor suppressor gene has been described to be responsible for the cancer-prone Peutz–Jeghers syndrome. Somatic mutation, chromosomal deletion or hypermetylation of *STK11* have been detected in gastric cancer and the combination of inactivating alterations of *TP53* and *STK11* has been demonstrated to work together in the acceleration of tumorigenesis in an animal mouse model [30,37]. Recently, mutation of *STK11/LKB1* has been described to induce loss of cell growth inhibition in squamous cell carcinoma cell lines [22]. Our data showed an association between loss of *STK11* and advanced stages and lymph node positivity in larynx and pharynx carcinomas. At the moment, the function of this serine/threonine kinase has been related to the AMP-activated protein kinase (AMPK). In response to energy stress, *STK11* would stimulate the AMPK signaling cascade to protect cells from apoptosis [7].

The *N33* gene is located at 8p22 and encodes a protein that is expressed in most non-lymphoid cells including tissues such as prostate, lung, liver and colon [18]. Loss of heterozygosity of chromosomal region 8p22 is known to occur frequently in epithelial tumors and loss of expression of two tumor suppressor genes located in these chromosomal region (*N33* and *EFA6R*) have been related to poor survival in ovarian carcinomas [24]. In non-small cell lung cancer, microsatellite instability of 8p was associated to lymph node positivity, squamous differentiation and LOH at *TP53* [39]. In addition, loss of 8p has been related to metastatic prostate cancer [20] and correlated with poor survival in HNSCC [3]. Our data confirm the relationship between loss of *N33* and advanced stage and positive lymph node and, in conclusion, a worse impact on larynx–pharynx carcinoma survival.

In summary, we propose that *N33* and to a lesser extent *STK11* and *TP53*, could have a role in the development of lymph node metastasis, which determines a poor survival in larynx and pharynx squamous cell carcinomas. Further studies are necessary to confirm their possible prognostic value and therapeutic applications.

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