

**Title: Genetic model of transformation and neoplastic progression in laryngeal epithelium**

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## ABSTRACT

**Background:** To analyse genetic alterations in the transformation-progression model of larynx tumors.

**Methods:** Copy number changes of 37 genes were analyzed by MLPA in 94 tissue samples.

**Results:** In the smoker normal mucosa group *TP53* loss was predominant, while in the precursor lesions *CDKN2A* loss and *CDKN2D* gain were most frequent. Precursor lesions with progression presented *CTNNB1* loss. In the carcinoma group the most common changes were *CDKN2A*, *MLH1*, *CTNNB1*, *CASP6* losses and *RECQL4*, *CCND1*, *EMS1* gains. Positive lymph node primary tumors were related to *TP53*, *IL1A*, *RBI* losses and *STK11* gain. The lymph node metastases differed from their corresponding primary tumor in *LMNA*, *RECQL4* and *IGF1R* losses, and *N33*, *CDKN2D* gains.

**Conclusions:** Genetic changes and new key genes were found associated to specific steps of transformation-progression. We included new steps, not presented in the classical models: normal mucosa tobacco exposed, positive lymph node primary tumor and corresponding lymph node metastases.

## INTRODUCTION

It is well established that the accumulation of genetic alterations is the basis for progression from normal cells to carcinoma, also called multistep carcinogenesis. This occurs in many solid tumors, including head and neck squamous cell carcinoma (SCC). The number of genetic alterations generally increases with the level of malignancy, and several theories have been proposed to explain this phenomenon.

The first genetic progression model in head and neck SCC was described in 1996. Califano et al.<sup>1</sup> established a correlation between genetic changes identified by loss of heterozygosity (LOH) and phenotypic changes of the normal mucosa, hyperplasia, dysplasia, carcinoma *in situ* and invasive carcinoma. This model, which supposed the beginning of a very interesting focus of investigation, was criticized because only 10 genetic events were considered, all exclusively related to loss of genetic material. Besides, some clinical factors and histopathological changes of the mucosa, such as tobacco exposure, laryngeal intraepithelial neoplasm (LIN) or lymph node metastases (LNM) were not taken into account. A second model was proposed by Braakhuis et al.<sup>2</sup>, based on the concept of 'field cancerization' in the upper aerodigestive tract (UADT). The study of LOH of *TP53* and *CDKN2A* in apparently normal tissues helped to distinguish second primary tumors from second field tumors or local recurrences. It has also been suggested that a stem cell, located in the basal cell layer of the mucosa, acquires genetic alterations and subsequently forms a 'patch', as a clonal unit of a stem cell with its daughter cells sharing the same DNA alterations<sup>3,4</sup>. This model of progression suggested that the initial genetic alteration is loss of 17p, followed by changes in 3p, 9p, 8p and 18q. Gain of 11q13 occurs once a carcinoma is developed. Genes that participate in metastatic spread remained unknown.

These two models do not exclude each other. On the contrary, they are related and are further complemented as knowledge about molecular mechanisms advances. ~~The~~ As causes of genetic instability and cellular immortality have been claimed ~~to be~~ activation of telomerase, mutation of *TP53* or *CDKN2A* or DNA aneuploidy. Anomalous methylation has also been noted in a variety of tumor suppressor genes (*ECAD*, *DAPK*, *CDKN2A* and *MGMT*), whose inactivation may play a role in tumorigenesis<sup>5</sup>.

Our purpose is to add new information and expand the model of larynx from normal epithelium to metastatic squamous carcinoma. Apart from the steps described by the classic models, we added 'normal tobacco exposed mucosa', 'LIN', 'negative lymph node primary tumor' (N0 primary tumor), 'positive lymph node primary tumor' (N+ primary tumor) and 'LNM' steps. Our genetic analysis of multigenic gains and losses was preformed by Multiple Ligation-dependent Probe Amplification (MLPA).

## MATERIAL AND METHODS

### Patients and samples

This is a cross-sectional study initiated in January 2002 which includes a retrospective analysis of patients diagnosed from 1999 of precursor lesions (PL) and SCC of the larynx with or without LNM, and a prospective research of patients diagnosed between January 2002 and December 2006 of normal mucosa without risk factor and normal mucosa tobacco exposed. Inclusion of patients ended in December 2006 and the final analysis was performed 6 months later (June 2007).

The inclusion criteria were: 1) Access to a complete clinical history of the patient. 2) A sample of tissue in optimal conditions for genetic analysis. 3) No prior radiotherapy or systemic chemotherapy. 4) A written consent of the patient when informed of the conditions of the study according to current regulations.

Eighty-four patients were finally selected from two hospitals of the Principado de Asturias (Spain): Hospital Valle del Nalón and Hospital Universitario Central de Asturias. The study was approved by the Commission of “Investigación y Ensayos Clínicos del Hospital Valle del Nalón”, Asturias, Spain.

Patients were distributed in the following groups (Table I):

**‘Without risk normal mucosa’**: Six children with tonsillectomy, not exposed to risk factors. The samples were obtained from an area of the normal mucosa from the aryepiglottic fold and immediately stored in liquid nitrogen.

**‘Smoker normal mucosa’**: Eleven active adult smokers (more than 20 cigarette/day for at least 10 years) and 4 of them (36%) also exposed to alcohol. Samples were obtained from laryngeal macroscopically normal mucosa from the aryepiglottic fold and immediately stored in liquid nitrogen.

**‘PL’**: Thirty-one patients (leukoplakia, 20 cases; erythroplakia, 8 cases and mixed-edematous, 3 cases). Most of these lesions were glottic (27 patients, 87%) and 4 were supraglottic (13%). All patients underwent a laryngeal microsurgery and were diagnosed according to the WHO histological classification, obtaining the following results: 3 cases of hyperplasia/keratosis (10%), 11 cases of LIN 1 (35%), 8 cases of LIN 2 (26%) and 9 cases of LIN 3 (29%). After an average follow-up of 41 months (range: 1-130 months) 22 patients (71%) presented complete response, 4 (13%) were diagnosed of recurrence of PL and in 5 cases (16%) an invasive carcinoma had developed. The time to progression from a PL to a carcinoma was variable, with an average time of 66 months

(6-125). All patients were alive at the end of the study. All 31 samples proceeded from paraffin embedded tissue after histopathological analysis.

**‘SCC’:** Thirty-four patients with invasive larynx SCC (19 supraglottic (56%) and 15 glottic (44%)). Histological grade of tumor differentiation was G1 (11 patients; 32%), G2 (18 patients; 53%) and G3 (5 patients; 15%). According to the TNM classification, 8 patients presented stage I (24%), 11 stage II (32%), 4 stage III (12%) and 11 stage IV (32%). In 12 patients lymph nodes were involved (N+ primary tumor). In all patients a radical surgery was performed, followed by radiotherapy in 12 cases (35%). After an average follow-up of 45 months, 20 patients had died (59%), 7 because of a locoregional recurrence, 3 with distant metastases and 4 due to a second primary tumor. Six patients died of other causes. SCC samples were obtained from surgical resection specimens of non-necrotic tumor areas and immediately stored in liquid nitrogen after surgery.

**‘LNM’:** Twelve corresponding LNM samples from the group of ‘SCC’ (N+ primary tumor), obtained at the same time as the primary tumor. All 12 samples were collected from paraffin embedded tissue after histopathological analysis.

In summary, 82 patients and 94 samples (43 from paraffin embedded tissue and 51 from frozen tissue) were studied.

### **MLPA analysis**

MLPA was performed as described in detail previously<sup>6,7</sup>. We used the probe mixture ‘P084 HNSCC-1’ (MRC-Holland, Amsterdam, The Netherlands). This mix was chosen to enable the analysis of 42 genes located in those chromosomal regions that according to literature may be involved in head and neck SCC.

For PL and LNM samples, three paraffin sections of 40 µm were carefully microdissected by the example of a hematoxylin-eosin stained 4 µm section and isolation of DNA was performed with fast spin-column (QIAamp®)(Qiagen GMBH, Hilden, Germany) as described before in detail<sup>7</sup>. For the normal mucosa groups (without risk and smoker), absence of histopathological lesion was checked using a hematoxylin-eosin stained 4 µm section. For the SCC cases, tumor purity was evaluated using a hematoxylin-eosin stained frozen section of the sample, and accepted when contaminating normal cells such as stroma and infiltrating lymphocytes were present at 20% or less. SCC tumor DNA was purified using phenol-chloroform extraction following a standard DNA isolation protocol.

Normal DNA from blood of 11 healthy donors and 10 paraffin blocks of negative lymph nodes were used as a control for tumor DNA samples from respectively fresh-frozen and paraffine tissues. Each sample was analyzed at least twice. For every gene, the relative copy number was calculated by dividing the average relative peak area

of the sample by the median relative peak area of the normal sample [poner referencia nuestra cellOncol 2005 y quitar referencia Moerland!](#)). We observed that a MLPA experiment analyzing 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.

### Statistical analysis

The statistical analysis was carried out using the Statistical Package for the Social Sciences version 12.0 for Windows (SPSS® Inc. Illinois, USA). Student's test was applied for quantitative parameters, and Pearson and Fisher Exact Chi-square for qualitative parameters. Chi-square McNemar test with Yates correction was used to evaluate differences between pairs of positive lymph node primary tumor and LNM. *P*-values  $\leq 0.05$  were considered significant.

## RESULTS

Five of 42 MLPA probes did not work properly and were excluded from analysis (*CDKN2B*, *BLM*, *TP53*-exon 2, *MADH2* and *MYBL2*). Results obtained for each group were the following:

**'Without risk normal mucosa'**: No genetic changes were detected.

**'Smoker normal mucosa'**: Copy number losses were identified in 7 out of 11 cases (64%). The most common alterations were loss of *TP53* (45%) and *CDKN2D* (27%) (Fig 1).

**'Precursor lesions'**: Twenty-seven out of 31 samples presented losses (87%) and 16 presented gains (51%). Four cases (13%) did not present any alteration. Most frequent alterations were loss of *CDKN2A* (58%), *A1651963* (26%), *EMS1* (26%), *BCL2* (26%) (Fig.1), and gain of *CDKN2D* (26%), *CTBS* (23%), *BCL2L1* (23%), *N33* (16%) and *MFHAS1* (13%). The number of genes with alterations was higher in PL than in the smoker group. Loss of *CDKN2A* occurred more frequent in PL compared to the smoker group ( $p=0.001$ ) (Fig. 1). However, loss of *TP53* was significantly associated to the smoker group compared to the PL ( $p=0.02$ ) (Fig. 1).

Loss of *CTNNB1* was detected in 4 out of 5 PL patients with progression to invasive larynx carcinoma and in 2 out of 26 PL patients without progression ( $p=0.002$ ) (Fig. 2).

**'Squamous cell carcinomas'**: All cases presented genetic changes. Most recurrent alterations were loss of *CDKN2A* (74%), *MLH1* (62%), *CTNNB1* (53%), *CASP6* (47%), *IL18* (44%), *IGSF4* (41%), *TP53* (38%), *CDH2*

and *BCL2* (35%) (Fig 3). Most frequent gains were *RECQL4* (50%), *CCND1* (47%), *EMSI* (44%), *PTP4A3* (41%), *LMNA* (35%). *CCND1* and *EMSI* also presented amplifications (32 and 24% respectively).

Genetic losses as well as gains were more frequent in SCC than in PL (Fig. 3 and 4). Only losses of *CCND1* and *EMSI* and gain of *N33* were observed in a higher number in PL group but this did not reach significance (Fig. 3 and 4).

N0 and N+ primary tumors presented different profiles of genetic changes. Losses of *TP53* ( $p=0.001$ ), *IL1A* ( $p=0.004$ ), *RBI* ( $p=0.031$ ) and gain of *SKT11* ( $p=0.031$ ) were significantly more frequent in metastatic primary tumors.

**‘Lymph nodes metastases’:** The most frequent alterations were losses of *CTNNB1* (83%), *NRAS*, *MFHAS1*, *CDKN2A*, *IL18* and *IGF1R* (50%) and gains of *PTP4A3* (58%), *CCND1*, *EMSI* (50%), and *SKT11* (42%) (Fig. 5). Genetic alterations occurring more frequent in LNM as compared to their original primary tumor were losses of *LMNA* (5 cases LNM/ 0 case N+ primary tumor), *RECQL4*, *IGF1R* (both with 4/0) and *MFHAS1* (4/1), and gains of *N33* (4/0) and *CDKN2D* (3/0) but this did not reach significance.

## DISCUSSION

Models of progression are important for the genetic analysis of cancer. Tumoral transformation-invasion follows a sequence of steps that in classical models included normal mucosa > precursor lesions > invasive tumor<sup>1,5</sup>. Our model extends the classic models with the steps of normal mucosa tobacco exposed, negative and positive lymph node primary tumor and LNM.

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MLPA is an available technique to study genetic alterations in fresh samples as well as in paraffin samples. Our interpretation of the MLPA data is in accord with Moerland et al. who found a 98% concordance with data obtained by FISH spotcounting, both performed on paraffin tumor material<sup>8</sup>.

MLPA is an available technique to study genetic alterations in fresh samples as well as in paraffin samples (ref Nygren AOH et al. Nucleic Acids Res. 2005 Aug 16;33(14): e128. doi: 10.1093/nar/gni127. Añadir). Using control DNA samples from the same source of tissue as the study sample (fresh or paraffin) both could be interpreted applying the relative copy number boundaries as indicated in material and methods. Comparison of MLPA data with other techniques like FISH or CGH has shown concordance results (ref Moerland and ours CGH article Cell Oncol2005).

Comparisonn

~~We found that when the DNA of normal controls was from the same source of tissue (paraffin blocks or fresh) as the test samples, both could be interpreted using the relative copy number boundaries as indicated in material and methods.~~

For the without risk normal mucosa group, samples from children were selected to avoid possible genetic alterations ~~damage possibly caused~~ by passive tobacco exposure. No genetic alterations were found in the first group. We have selected samples from children with a very short time of exposure to risk factors (marta: is deze zin dubbel met de eerste zin van deze paragraaf?). Our results are in agreement with other authors. ~~They who~~ also used samples from the aryepiglottic fold as control and they did not find genetic changes by LOH analysis in microsatellites D9S171 (9p21), D3S1007 (3p21.3-22) and D3S1228 (3p14)<sup>9</sup>.

In the smoker normal mucosa group, all cases had tobacco exposure (at least 20 cigarettes/day and more than 10 years of smoking) and they were active smokers at the moment of these study. In this group, the most frequent genetic alterations occurred in *TP53* and *CDKN2D*, both ~~as a loss being deletion~~. Expression of p53 has been observed before in normal mucosa samples from smokers by immunohistochemistry, although this expression may be the result of a complex regulatory mechanism<sup>10</sup>; it has been suggested that in a first step ~~an increase of~~ normal protein ~~occurexpression increases~~, in a second step ~~there is a co-expression of~~ normal and mutated protein ~~are co-expressed~~, and finally only mutated protein would be expressed. This phenomenon subscribes the theory that tobacco acts as a mutagenic agent in UADT, and mutation of *TP53* is present in adjacent cellular groups as a *patch*, until the cell cycle dysregulates because of dysfunction of p53 protein. *CDKN2D* has been described as a tumor suppressor gene involved in cell cycle regulation and kinase activity. Deletions have been associated to many tumors<sup>2</sup>.

Our series include 31 PL and 34 SCC related to tobacco and alcohol exposure (Table I). This combination is typical for ~~patients carrying~~ this kind of tumors, ~~which occur~~ most frequently in male patients about 60-70 year old, although the number of female patients is increasing in the last decades due to changes in life-style.

The PL group presented gains and losses, with predominant loss of *CDKN2A* (Fig. 1), which has been described in literature as an early event<sup>11</sup>. Deletion of 9p21 was found in brushings of oral lesions, saliva or spittle, and has been used to identify high risk patients. It is an early marker of LIN and patients should be advised to quit smoking when deletion is found.

We have found that loss of *CTNNB1* differentiated between PL without progression and PL with progression, and this might be used as a prognostic indicator to adequate treatment and follow-up, however, further studies would be necessary to validate this observation. This gene, coding the  $\beta$ -catenin protein that together with E-cadherin participates in cellular adherence, forms intercellular junctions and maintains the epithelial structure. Several publications consider loss of activity of cadherins as a early step for SCC, but until now it had not been described in PL<sup>12</sup>. Recently, osteopontine and its receptor CD44v6 (adhesion molecule) have also been implicated in LIN and progression by immunohistochemistry analysis<sup>13</sup>.

The SCC group showed many genetic abnormalities. The most common were losses of *CDKN2A* and *MLH1* and gains of *CCND1*, *EMS1*, *RECQL4* and *PTP4A3*, with also amplifications in *CCND1* and *EMS1* (Fig. 3 and 4).

~~Results~~ These results are similar to those previously published by Comparative Genomic Hybridization (CGH) and Fluorescence In Situ Hybridization (FISH) in SCC<sup>14,15</sup>.

Gains and amplifications of 11q13 (*CCND1*, *EMS1*, *INT2*) are generally found in 50-60% of head and neck SCC<sup>16</sup>, and this is in agreement with our data.

Loss of *CTNNB1*, a ~~significant~~ alteration significantly associated to PL with progression to carcinoma in our study, was even more frequent in SCC, suggesting an accumulative alteration along the transformation and progression model (Fig. 2 and 3).

Many of the progression models consider PL and SCC as consecutive steps but only 7-8% of head and neck SCC are first diagnosed as PL. ~~Our~~ The MLPA genetic patterns in our study differed between PL and SCC, suggesting that PL might not necessarily be a precursor of SCC. Gains of *PTP4A3*, *RECQL4A*, *CCND1* and *EMS1* were ~~more~~ common in the carcinomas but in PL we observed losses of *CCND1* and *EMS1*, concurring with early loss of heterozygosity at 11q13 as described by Califano et al<sup>1</sup>. ~~However, it~~ it may be speculated that those PL that harbouring loss of *CTNNB1* are precursor to SCC.

Several biological mediators have been implicated in the metastatic process, including cellular adhesion molecules, proteolytic enzymes, growing factors, metastasis suppressor genes and lymphokines receptors<sup>17</sup>. We have observed different genetic patterns between N0 and N+ primary tumors with losses in *TP53*, *IL1A* and *RBI* and gain of *STK11*, associated to N+ primary tumors. P53 protein is a key regulator of the cell cycle and apoptosis functions. Genetic loss of *TP53* has been detected in head and neck SCC and also in many other tumor types<sup>18</sup>. *IL1A*, a member of the tumor necrosis factor (TNF) receptor family, has an important role in the immune

response regulation and the programmed cell death in ovary epithelial cells<sup>19</sup>. Alterations of the retinoblastoma gene (*RBI*) with additional genetic changes of *TP53* have been described in osteosarcoma and small-cell lung carcinoma<sup>20</sup>. According to chromosomal patterns of gains and losses, it has been possible to identify metastatic and non-metastatic primary tumors by CGH. Thus, gains of 5p, 6p and 7p were seen in non-metastatic carcinomas and losses of 7q, 10q, 11p, 11q, 15q, 20p, 19q and 20q in metastatic tumors<sup>21</sup>. Using microarray expression [techniquesanalyses](#), *TIMP-1*, *COL11A1* and *SERPINB2* genes were demonstrated to be overexpressed in metastatic tumors<sup>22</sup>, but some authors disagree<sup>23</sup>.

Genetic changes [differed](#) between N+ primary SCC and the corresponding LNM ~~were different~~ (Fig. 5). Also in the literature, discordance of 35% ~~has been~~ [was](#) found between metastases and primary tumors by microsatellite analysis<sup>24</sup>. Our data confirm this, suggesting the dissemination of a particular subclone of the primary tumor toward lymph nodes. Clinical assessment of lymph nodes can be carried out by non-invasive techniques like fine needle puncture, as it is performed for the diagnosis of micrometastases with cytokeratin 5 and 14 by RDT-PCR<sup>25</sup>. Therefore, it would be most valuable to identify genetic markers for lymph node metastasis.

In figure 6 we [presented](#) the most frequent genetic alterations of each group, and also the key genes that make a difference in every step and might have importance for clinical practice or in therapeutic decision-making. The most important genetic alterations found along the different steps of transformation-progression model and their biological functions are summarized in table II.

In conclusion, we believe that MLPA is a useful multigenic analysis method for the description of a model of tumor progression in UADT. Information about an important number of genes can be obtained. MLPA is not a screening technique but may be used to guide molecular studies to specific/hotspots genes.

The expanded model of transformation and neoplastic progression in laryngeal epithelium is summarized in figure 6. In this model, results from normal tobacco exposed mucosa and corresponding LNM from the original N+ primary tumors are included. Genetic changes and new key genes were associated to these new steps in the transformation-progression from a normal tissue to a SCC and LNM. Our results may allow to better understand the genetic changes and pathways that are involved in larynx SSC carcinogenesis. We propose that this genetic model may serve as a guide for further molecular and [protein](#) expression studies. In the future, a reduced kit for DNA copy number and /or gene expression analysis might be developed to identify genetic changes in each step (tobacco normal mucosa kit, PL kit, LNM kit) in order to facilitate the translation into clinical practice.

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## FIGURES LEGENDS

Figure 1. Genetic losses in smoker normal mucosa (white bars) and precursor lesions group (dark bars). Significant differences associated to each group are indicated by arrows.

Figure 2. Genetic losses in PL without progression (white bars) and PL with progression group (dark bars). Loss of *CTNNB1* was significantly associated to PL with progression (indicated by arrow).

Figure 3. Genetic losses in PL (white bars) and SCC group (dark bars). Many losses were found in SCC.

Figure 4. Genetic gains in PL (white bars) and SCC group (dark bars).

Figure 5. Genetic losses in N+ primary tumors (white bars) and their corresponding LNM group (dark bars).

Figure 6. Genetic progression model by MLPA . Important genetic alterations in every step (-: loss, +: gain) and key genes between 2 steps (🔑) are shown.