Genetic profile of second primary tumors and recurrences in head and neck squamous cell carcinomas

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Abbreviations: MLPA: Multiplex Ligation-dependent Probe Amplification; HNSCC: head and neck squamous cell carcinoma; N0: negative lymph node; N+: positive lymph node.

ABSTRACT

Background: Second primary tumors and recurrences are an important problem in patients with head and neck squamous cell carcinoma. To determine the genetic changes in tumor samples in order to improve knowledge of tumor progression.

Methods: Copy number changes of 37 genes were analyzed by multiplex ligation-dependent probe amplification (MLPA) in 36 primary tumors and their corresponding 21 second primary tumors and 15 recurrences.

Results: *CCND1* and *EMS1* amplifications and gain of *BCL2L1 we*re the most common genetic alterations in the primary tumor, second primary tumor and recurrence samples. Gains of *ERBB2* and *PTPN1* were associated to recurrences.

Conclusions: Specific genetic profiles for each group have been found. Similarities between primary tumor and second primary tumor and dissimilarity between primary tumor and recurrence suggest that clinic-pathological criteria do not always accurately differentiate these entities. Genetic profiling may aid in the diagnosis and prognosis of these difficult cases.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) has a high incidence in Spain. Despite progress in diagnosis and treatment in the last decades, tumors are still diagnosed in late stages and the overall survival for HNSCC has not significantly improved[1]. Tumor recurrences as well as development of second primary tumors and distant metastases are the main factors that adversely influence the overall survival of these patients[2]. The rate for recurrences in patients with HNSCC varies from 16% to 70% especially in advanced tumor stages[2,3]. Although the incidence of tumor recurrences appears to decrease due to better locoregional control of the disease, it is still the main cause of death in patients with advanced HNSCC [3,4]. The incidence rate of second primary tumors in patients with HNSCC varies from 5% to 36%[2,5]. The development of second primary tumors is associated with decreased overall survival as it is the leading cause of treatment failure and death in patients with early stage HNSCC[6], but the prognosis of patients with second primary tumors is much better than those diagnosed with recurrences or distant metastases[5,7,8]. The risk of developing a second primary tumor is constant and therefore diligent surveillance over the patient's lifetime is important although there is disagreement on timing or assessment of follow up [2].

It is of clinical relevance to distinguish among a recurrence, a distant metastases and a second primary tumor. Clinical criteria used in practice are poorly defined and lead to confusion. Some authors have proposed that molecular techniques may enable to dissect the routes that lead to the development of further tumors after diagnosis and treatment of primary tumors[9,10]. Many studies have addressed the molecular basis of cancer development and genetic progression models have been proposed for various tumor types, including HNSCC. However, we still do not know the proximate causes of most cancers. It is now well established than an accumulation of genetic and epigenetic alterations are the basis for the

progression from a normal cell to a cancer cell, referred to as the process of “multistep carcinogenesis”[10,11].

The development of local tumor recurrences and second primary tumors has frequently been explained by the concept of “field cancerization”[9]. According to this hypothesis, multiple non-related precancerous lesions may exist adjacent to the original primary tumor, each with the capacity to form new tumors. A modified version of “field cancerization” defines a true second primary tumor as genetically independent of the “index lesion” or primary tumor. Alternatively, the term “second field tumor” is used when new tumors arise in the same precancerous field having genetic fingerprints similar to that of the primary tumor[5,10]. A competing biological model is clonal expansion. A second primary tumor may present with identical genetic markers as the primary tumor, indicating development from the same clone. Progenitor cells from the primary tumor are thought to spread, implant and grow at a new site. A recently proposed model to explain tumor progression is the "Cancer Stem Cell Network" hypothesis. This model defines a stem cell network as a pool of functionally related somatic stem cells that maintain the homeostasis and tissue repair of an anatomical region. In a stem cell network the somatic stem cells derived from a common embryonic stem cells share a specific molecular profile and maintain a high degree of cell-cycle synchronization. This model also implies the mobility of stem cells, which may migrate selectively from one niche to another within the same stem cell network, so these cells would create a “field of cancerization” with the shape and size of the stem cell network[11].

It would be useful to link molecular techniques with clinical practice in order to improve biochemoprevention, post-therapy surveillance protocols and diagnosis of HNSCC progression.

The aim of this study is to determine the genetic changes in second primary tumors and recurrences in relation to their corresponding primary tumor, in order to improve knowledge of tumor progression and to evaluate a possible application in early diagnosis and prognosis of second primary tumors and recurrences. “Multiplex Ligation-dependent Probe Amplification” (MLPA), a rapid, semiquantitative and high resolution technique, capable of detecting more than 40 different genes/DNA sequences in a single experiment, was used to study the gene copy number changes.

PATIENTS AND METHODS

This is a cross-sectional study initiated in December 2004 which includes a retrospective analysis of patients diagnosed from January 1983 and June 2004 of HNSCC with progression toward second primary tumor and recurrence. The final analysis was performed in August 2009.

The inclusion criteria were: 1) Access to a complete clinical history of the patient. 2) A tissue sample in optimal conditions for genetic analysis. 3) No prior radiotherapy or systemic chemotherapy and also no posterior radio- or chemotherapy treatment between primary tumor and recurrence or second primary tumor. 4) A written consent of the patient when informed of the conditions of the study according to current regulations.

Thirty-six patients with larynx or pharynx primary tumor were selected from two hospitals of the Principado de Asturias (Spain): Hospital Valle del Nalón/Valle del Nalón Hospital and Hospital Universitario Central de Asturias/University Central Hospital of Asturias. The study was approved by the Commission of “Investigación y Ensayos Clínicos del Hospital Valle del Nalón”, Asturias-Spain. “Research and Clinical Tests of Valle Del Nalón Hospital, Asturias, Spain”.

All patients underwent radical surgery. No radiotherapy or systemic chemotherapy was employed as complementary or radical treatment for the primary tumors in order to avoid misrepresenting genetic tumor profile. Thirty-five were smoker patients (97%) and 28 (78%) were also exposed to alcohol.

All clinico-pathological characteristics (localization, tumor stage, histological grade, lymph nodes, surgical margins, peritumoral dysplasia), are summarized in table 1.

During the follow-up, 21 patients (58%) development a second primary tumor and 15 patients (42%) a recurrence.

- Second primary tumor and corresponding primary tumor samples.

Twenty-one second primary tumors samples from the 21 patients with a larynx or pharynx primary tumor were collected. Second primary tumor was defined by standard criteria (Warren and Gates's criteria): both tumors were histologically malignant; the two cancers were anatomically separated by more than 2 cm of normal mucosa, without submucous invasion and the possibility that one tumor represented metastasis from the other was excluded [2,5]. Second primary tumor was further classified as synchronous when the diagnosis was simultaneous or within 6 months after the primary tumor, or as metachronous when it was diagnosed at least 6 months after the primary tumor [5].

Nineteen patients (90%) were male and two (10%) were female. The mean age was 57 years (range 39-76). In our study 19 patients (90%) had second primary tumors in the head and neck region and 2 patients (10%) in distant organs (1 lung, 1 esophagus).

The mean interval between the primary tumor and the second primary tumor was found to be 42 months (range 0-121). Metachronous second primary tumors occurred more often than synchronous second primary tumors (16 patients, 76% *versus* 5 patients, 24%). All

our synchronous second primary tumors were simultaneous. Two patients (10%) continued smoking and drinking after the treatment of the primary tumors.

The 21 primary tumors were originated in the larynx (12 patients, 57%) or in the pharynx (9 patients, 43%). Patients with second primary tumors had supraglottic larynx and oropharynx tumors (7 cases, 33% both of them), glottis (5 cases, 24%) and hypopharynx (2 cases, 9%). The primary tumors were all squamous cell carcinoma in different grades of histological differentiation: G1 (14 patients, 67%), G2 (4 patients, 19%), G3 (3 patients, 14%). The tumor stage at presentation was grouped as initial (I and II) in 13 patients (62%) and advanced (III and IV) in 8 patients (38%). Surgical margins were negative in 18 patients (86%), positive in 3 (14%) (Table 2: cases 2, 5 and 14), and 4 patients (19%) presented peritumoral dysplasia (Table 1). There were 17 patients (81%) with N0 (negative lymph node) and 4 (19%) with N+ (positive lymph node). In the second primary tumors one patient (5%) presented papillary carcinoma of the oropharynx. The stage of the second primary tumors was grouped as initial (I and II) in 13 patients (62%) and advanced (III and IV) in 8 patients (38%).

A follow-up was available for 0 to 121 months (median 28 months) after treatment of the primary tumor. The 5-year survival rate was 67% (14 patients). During the follow-up, 7 patients (33%) died due to the tumor.

- Recurrences and corresponding primary tumor samples.

Fifteen tumor recurrence samples from the 15 patients with larynx or pharynx squamous cell carcinoma primary tumors were collected. We define tumor recurrence as the process of tumor development that occurs at a distance $\leq$ 2 cm from the initial tumor and within 3 years after the primary tumor[5,12]. Recurrence is classified as local (in the same anatomic site as the primary tumor), locoregional (adjacent, deep or affected regions with

cervical extension), and stomal (infiltrating the tracheal stoma)[2,3]. Recurrences were also classified as early when they were diagnosed within 6 months of the primary tumor, or late when they were diagnosed more than 6 months after the primary tumor[12].

All patients were male. The mean age was 61 years (range 41-71). In our study, the percentage of locoregional, local and stomal recurrences were 53% (8 patients), 33% (5 patients) and 13% (2 patients), respectively. The interval between the primary tumor and the recurrence appearance was 12 months (range 1-37). After the treatment of the primary tumor all patients stopped alcohol and tobacco consumption.

The primary tumors were localized in the larynx (8 patients, 53%) or in the pharynx (7 patients, 47%) (table 2). They were all squamous cell carcinoma in different grades of histological differentiation: G1 (6 patients, 40%), G2 (8 patients, 53%), G3 (1 patient, 7%). The tumor stage at presentation was grouped as initial (I and II) in 6 patients (40%) and advanced (III and IV) in 9 patients (60%). Surgical margins were negative in all cases (100%), whereas 3 patients (20%) presented peritumoral dysplasia. There were 9 patients (60%) with N0 and 6 patients (40%) with N+. A follow-up was available for 0 to 115 months (median 31 months) after treatment of the primary tumor. The 5-year survival rate was 20% (3 patients). During the follow-up, 11 patients (73%) died from the tumor and one (7%) died from concomitant disease.

-MLPA analysis.

Samples of primary tumors, second primary tumors and tumor recurrences were obtained from surgical resection specimens or biopsies of non necrotic tumor areas and immediately stored in liquid nitrogen after surgery or from paraffin-embedded tissue at the Department of Pathology after histopathological analysis.

Tumor DNA was extracted by deparaffination steps in xylene, wash steps in methanol and PBS buffer, incubation overnight in 1M sodium-thiocyanate and, finally, digestion and purification using Qiagen columns (Qiagen GmbH, Hilden, Germany). 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) and used as a control for samples.

MLPA was preformed as described in detail previously using the probe mixture "SALSA P084 HNSCC1" (MRC-Holland, Amsterdam, the Netherlands (www.mlpa.com))[13]. This probe mixture contained 42 probes for specific genes located in those chromosomal regions that according to literature may be involved in HNSCC. 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 98ºC for 5 min and hybridized with the MLPA probe mixture at 60ºC for 16 hours. Ligation of the two parts of each probe was performed by a thermostable ligase. All probe ligation products have the same end sequences and were amplified by PCR using the same primer pair for 60ºC 1 min, 33 cycles of 95ºC 30 sec, 60ºC 30 sec and 72ºC 1 min, followed by 20 min at 72ºC and kept cold at 4ºC. The products were subsequently analyzed on an ABI Prism 3100 sequencer and by GeneScan v3.7 software (Applied Biosystems, Warrington, UK).

Ten control experiments using normal DNA from 5 different donors were used to calculate median reference values and the standard deviations for every probe. Each tumor sample was analyzed 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 an MLPA experiment analyzing 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, whereas values higher than 1.2 would be gains and values over 2.2 or higher would count as amplifications.

-Statistical analysis.

The statistic analysis (SSPS for window, release 15.0, Chicago, IL, USA) was carried out using Student's test and Fisher Exact Chi-square test. Differences between genetic copy number changes of primary tumors and corresponding second primary tumors and tumor recurrences were compared using McNemar´s test. Survival estimation was analyzed by Kaplan-Meier statistics with the Log rank test. Losses, gains and amplifications from MLPA results were tested for correlation with survival. *P*-values < 0.05 were considered significant.

Finally, we compared the genetic profile obtained by MLPA of each primary tumor and the corresponding second primary tumor or recurrence. If two profiles showed more than 50% of copy number alterations, this was considered "high similarity"; if common copy number alterations were between 25 to 50%, "uncertain similarity"; and if less than 25%, "low similarity".

RESULTS

Thirty-seven out of the 42 probes in the MLPA kit worked reproducibly in our hands, 5 probes did not work properly and were discarded of the analysis: *TANK, CDKN2B, TP53* (exon 2), *IGF1R, MYBL*. Gene copy number alterations were found in all samples.

- Second primary tumor and corresponding primary tumor

In the 21 patients with primary tumors and their corresponding second primary tumors, the 21 primary tumors showed losses of *CDKN2A* (10 cases, 48%); *MLH1* and *AI651963* (7 cases, 33%); *CTNNB1* and *CASP6* (6 cases, 28%) (Figure 1). Gains of *BCL2L1* were found in 10 cases (48%). We also found gains of *CCND1* and *EMS1* (9 cases, 43%) (Figure 2). We detected 15 amplifications: *EMS1* (6 cases, 28%), *CCND1* (4 cases, 19%); *MLH1*, *CTSB*, *N33*, *ERBB2* and *PTPN1* (1 case, 5%).

The most common alterations detected in the 21 second primary tumors were losses of *CDKN2A* (14 cases, 67%), *TP53* (6 cases, 28%) (Figure 1), and gains of *CCND1* and *EMS1* (9 cases, 43%), *BCL2L1* (8 cases, 38%) and *N33* (7 cases, 33%) (Figure 2). We detected 14 amplifications: *CCND1* and *EMS1* (5 cases, 24%), *IL1A*, *CTSB*, *CDKN2A* and *ERBB2* (1 case, 5%). Gains of *IL1A*, *IL2*, *N33*, *RECQL4*, *AI651963*, *RENT2*, *IL18*, *BRCA2*, *CDH2* and *MADH2* were more frequent in second primary tumors compared to their corresponding primary tumors although this was not statistically significant (Figure 2).

Comparisons of genetic profiles (primary tumor and corresponding second primary tumor) showed that 2 pairs (10%) had “high similarity”, 7 pairs (33%) had “uncertain similarity” and 12 pairs (57%) had “low similarity” (Table 2).

- Recurrences and corresponding primary tumor

In the 15 patients with primary tumors and their corresponding recurrences, the 15 primary tumors found losses of *CDKN2A* (7 cases, 47%), *CDH2* (7 cases, 47%), *AI651963* (6 cases, 40%) and *MFHAS1*, *IL18* and *BRCA2* (5 cases, 33%), (Figure 1). We also observed gains of *LMNA* and *BCL2L1* (7 cases, 47%), *CCND1* and *EMS1* (6 cases, 40%) and *CDKN2D* (5 cases, 33%), (Figure 2). We detected 20 amplifications: *CCND1* and *EMS1* (6 cases, 40%), *BCL2L1* (3 cases, 20%), *LMNA*, *MFHAS1*, *PTP4A3*, *REQL4* and *CDKN2D* (1 case, 5%).

The most common alterations detected in the 15 tumor recurrences were loss of *IL18* (7 cases, 47%), *AI651963* (6 cases, 40%), *CASP6* and *RENT2* (5 cases, 33%) (Figure 1), and gains of *LMNA* (11 cases, 73%), *EMS1* and *BCL2L1* (9 cases, 60%), *CCND1* and *PTPN1* (8 cases, 53%), *MFHAS1*, *CTSB* and *ERBB2* (5 cases, 33%) (Figure 2). We detected 17 amplifications: *CCND1* and *EMS1* (6 cases, 40%), *CTBS* and *ERBB2* (2 cases, 14%), *LMNA* (1 case, 7%). Gains of *ERBB2* (p=0.041) and *PTPN1* (p=0.019) were more frequent in recurrences compared to their corresponding primary tumors (Figure 2).

Comparisons of genetic profiles of primary tumors and corresponding recurrences showed 2 pairs (13%) with "high similarity", 6 pairs (40%) with "uncertain similarity" and 7 pairs (47%) with "low similarity" (Table 3).

- Second primary tumor versus recurrences

When we compared the genetic profiles of second primary tumors and recurrences many differences were found (Figures 1 and 2). Losses of *CDKN2A* (p=0.004) and *TP53* (p=0.01) and gain of *N33* (p=0.04) were significant related to second primary tumors and losses of *IL18* (p=0.004), *MFHAS1* (p=0.048), *AI651964* (p=0.021), *RENT2* (p=0.048) and gains of *LMNA* (p=0.001), *PTPN1* (p=0.003), *CTSB* (p=0.048) were significant associated to recurrences (Figures 1 and 2).

We did not find any statistically significant correlation between the genetic alterations and clinico-pathological characteristics such as stage, tumor grade, lymph node, margins and dysplasia (Table 1). In addition, survival was not related to primary tumors, second primary tumors or recurrences localization and time to develop a second primary tumors or recurrences (Table 2 and 3).

DISCUSSION

HNSCC are known to harbor a very large number of chromosomal aberrations. These molecular alterations already occur in the early phases of carcinogenesis and continue to accumulate progressively, leading to malignancy. This makes it difficult to know which ones may be related to clinical behavior[10,14]. The development of second primary tumors and recurrences is associated with decreased overall survival as it is the leading cause of treatment failure and death in patients with early and advanced stage of primary HNSCC[2,3,5].

The aim of this study was to determine the genetic changes in the primary tumors in relation to their corresponding second primary tumors or recurrences. Samples were collected from patients that had never received radiotherapy or chemotherapy treatment, in order to avoid unspecific genetic alterations by the treatment. This requirement decreased the number of samples that can be collected for this study, especially for the larynx and pharynx tumors that are normally diagnosticated in advanced stages.

The MLPA kit P084 employed in this study enables the simultaneous analysis of several genes located in chromosomal regions that according to literature may play a role in HNSCC. We are aware of the possibility that the selected genes in the kit may not be the ones that triggered the occurrence of a certain gain or loss of the chromosomal region and that a neighboring gene may be more important. In HNSCC, frequent losses have been found by CGH at chromosome arms 3p, 8p, 11qter and 18q, and gains at 3q, 8qter, 5p and 11q13 [15]. Unfortunately, our MLPA kit contained no probes for genes at 3q, 5p or 18p, regions that are known from literature and previous reports from our group to frequently present copy number gains in HNSCC[15-17].

- Second primary tumor and corresponding primary tumor

In the group of 21 patients with primary tumors that later developed a second primary tumor, the most frequent losses found in the primary tumors samples were located at *CDKN2A* (9p21), *MLH1* (3p22) and *AI651963* (10p14) (Figure 1). These losses are in agreement with the literature that indicated deletions in both chromosomal regions 9p and 3p as an early event in the HNSCC development[10,18]. Loss of *MLH1* and *CDKN2A* play an important role in laryngeal carcinoma development and progression[19]. Both LOH and aberrant methylation contribute to *MLH1* and *CDKN2A* inactivation in laryngeal carcinoma, and are associated with a less advanced stage of larynx tumors[20].

Loss of *AI651963* (10p14) was found frequently in the primary tumors (Figure 1). *AI651963* is not a real gene; it is cDNA clone that hybridizes to 10p14 region. No genes were described at 10p14, and there is not information about the relationship between this region and HNSCC. This probe could be related to an unknown gene, near to *AI651963*, and involved in head and neck carcinogenesis.

Other common genetic alterations detected in the 21 primary tumors were losses of *CTTNB1* (3p22) and *CASP6* (4q25) (Figure 1). Beta catenin (*CTNNB1* gene) is an adherent junction protein, critical for the establishment and maintenance of epithelial layers, communicating signals to neighboring cells and anchor the actin cytoskeleton. Alterations in this gene might lead to tumor invasion and also enhance cellular migration. Deletions of 3p have been described in precursor lesions, and therefore this alteration is interpreted to be an early marker of carcinogenesis[10,18]. *CASP6* plays a crucial role during apoptosis. Decreased expression of *CASP6* has been related to the pathogenesis of gastric and colorectal cancers[21], but not to HNSCC. The genetic alterations at 4q have been related to have a high probability of progression into invasive carcinoma[22].

Loss of *CDKN2A* was also the most common genetic alteration in the 21 second primary tumors (Figure 1). Although loss of *TP53* (17p13.1) was another frequent alteration

in second primary tumors, loss and gain of this gene were described as shared genetic alterations between primary tumors and second primary tumors. Mutation of *TP53* has been suggested to be one of the earliest events giving rise to a field of cancerization with malignant potential[10,23]. The expression of p53 protein in HNSCC has been associated with earlier development of both recurrences and second primary tumors. Nonfunctional p53 results in advanced disease and poor prognosis of patients suffering HNSCC[24]. Our findings are in agreement to the literature.

BCL2L1 (20q11.1) was one of the shared genetic alterations between primary tumors (10 patients, 48%) and second primary tumors (8 patients, 38%). Both primary tumors and second primary tumors showed gain of *BCL2L1* in our study (Figure 1). That is according to the finding in the literature with upregulation of *BCL2L1* in advanced disease and poor prognosis of patients suffering HNSCC[24].

Gains and amplifications of *CCND1* and *EMS1* (11q13) were common genetic alterations in primary tumors and second primary tumors (Figures 2). Amplification of 11q13 has been described in relation to late events in the HNSCC development[25]. *CCND1* is related to the malignant clone selection in the "field of cancerization" and to worse prognoses in HNSCC patients and its amplification is associated with metachronous tumors [26]. Amplification of *EMS1* but not of *CCND1* correlated to advanced stage of the tumor and in this study many patients were diagnosed at late stages in primary tumors (7 patients, 33%) and second primary tumors (9 patients, 44%) (Table 1). This confirms that amplification of *EMS1* occurs independently from *CCND1* amplification as has been found in other studies on HNSCC[27].

Previous reports of our group have described gain and amplification of *CCND1*, and *EMS1*, and loss of *CDKN2A*, just as we have found in this study[10,28]. Nevertheless, loss of *AI651963* and gain of *BCL2L1* had not been described in relation to HNSCC until

now.Unfortunately, we could not find literature addressing the relation between primary tumors and their corresponding second primary tumors comparing the genetic patterns of individual tumors by MLPA in order to discuss our results.

Comparisons of genetic profiles showed “low similarity” in 12 of the 21 pairs (57%) and seven pairs (33%) were “uncertain similarity” (Table 2). Interestingly there were 2 pairs (10%) with “high similarity” (Table 2). In figure 4, example B we show the genetic profile obtained by MLPA of one of the two cases with “high similarity”(case number 6, in table 2). It is a patient with a glottic laryngeal squamous cell carcinoma T3N0M0G1R0 (stage III), with a laryngectomy surgery and a bilateral neck dissection. After 78 months the patient developed a tumor progression at the nasal cavity with the clinical characteristics of a T3N0M0G2R2 second primary tumor (case 6 from table 2). The MLPA genetic profile showed a “high similarity” between the primary tumor (Figure 4B1) and the second primary tumor (Figure 4B2). The similar genetic profile would support that it was a tumor recurrence or a distant metastases rather than a second primary tumor. The other “high similarity” case was diagnosed of synchronous second primary tumor (case 14, table 2). The primary tumor was located in the oropharynx and the second primary tumor in the glottis. It could be possible that this second primary tumor was a second field tumor originating from a field of cancerization.

- Recurrences and corresponding primary tumor

In the 15 patients with primary tumors that later developed a tumor recurrence, the most frequent losses were of *CDKN2A* (9p21) and *CDH2* (18q11.2). Loss of 9p21 has been described as an early event in head and neck cancer development and loss of 18q has been associated with the later stages of carcinogenesis[18].

A few altered genes analyzed in our MLPA study have not yet been related to HNSCC. For instance losses of *BRCA2* (13q12.3) (frequent in the 15 primary tumors), losses

of *CASP6* (4q25) and *RENT2* (10p14) (frequent in recurrences), gains of *LMNA* (1q21.2) (found in both primary tumors and recurrences, although more frequent in recurrences), gains of *PTPN1* (20q13.1) (frequent in recurrences) or gains of *CREM* (10p12.1) (found in both primary tumors and recurrences) (Figure 1 and 2). Although losses of *CASP6* were observed in our study, *CASP6* has never been related to HNSCC. However, genetic alterations at 4q have been associated to a high probability of progression into invasive carcinoma[21].

Other genes have already been associated with HNSCC such as losses of *IL18* (11q23.1) (found in both primary tumors and recurrences, although more frequent in recurrences), *AI651963* (10p14) and *MFHAS1* (8p23.1) (found in both primary tumors and recurrences) also related with patients with moderate and poorly differentiated carcinomas, *MLH1* (3p22.1) (quite common in recurrences), *RB1* (13q14.2) (quite common in recurrences), or gains of *CTSB* (8p22) and *PTP4A3* (8q24.3) (frequent in both groups) (Figure 1 and 2)[28].

Losses and gains of *TP53* and *BCL2* were other recurrent genetic alterations in both primary tumors and recurrences, and this is according to the literature (Figure 1 and 2) [24].

Both primary tumors and recurrences showed gains of *BCL2L1* (20q11.1) in our study. That is according to the following findings in the literature: up-regulation of *BCL2L1* results in advanced disease and poor prognosis of patients suffering HNSCC[24]. Many patients (47%) included in the study were diagnosed at advanced stage (III-IV) (Table 1), specially recurrences (67%).

We described gains of *CDKN2D* (19p13) in both primary tumors and recurrences. *CDKN2D* has been described as a tumor suppressor gene involved in cell cycle regulation and found to be deleted in many tumors[29]. However, we found gains instead of losses. A possible explanation is that this chromosomal region carries an oncogene, not included in this MLPA assay.

Gains and amplifications of *CCND1* and *EMS1* (11q13) were recurrent genetic alterations in primary tumors and recurrences (Figure 2)[10,25,27].

Gains of *ERBB2* (17q21.1) and *PTPN1* (20q13.1) were more frequent in recurrences compared to primary tumors so they could be useful to distinguish between primary tumor and recurrence (Figure 1).

Comparisons of genetic profiles showed 2 pairs (13%) with "high similarity" (Table 3), 6 pairs (40%) "uncertain similarity" and there were 7 pairs (47%) with "low similarity" which is surprising thinking that primary tumor and recurrences are very clonally related. In figure 4 example A, the genetic profile obtained by MLPA of one case with "low similarity" is showed (case number 7 in table 3). Primary tumor comes from a supraglottic laryngeal squamous cell carcinoma T3N2bM0G2R0 (stage IV), with a laryngectomy surgery and a bilateral neck dissection. After 4 months, the patient developed a tumor progression at the same supraglottic laryngeal localization and it was defined as a TxN+M0G2R2 locoregional tumor recurrence (case 7 from table 3). MLPA results showed a "low similarity" genetic profile between the primary tumor (Figure 4A1) and the recurrence (Figure 4A2). This low genetic similarity would be more in accordance with a second primary tumor than with a recurrence as it was classified. In this case, the lesions are initially genetically related ~~in localization~~ but diverge in a later stage so, some markers are similar and others are different.

- Primary tumor, second primary tumor and recurrences

The finding of common genetic alterations (gains and amplifications of *BCL2L1*, *CCND1* and *EMS1*) between primary tumors, second primary tumors and recurrences seems to suggest a "genetic fingerprint" for HNSCC (Figure 3). However, tumor recurrences, the most advanced phase of cancer development, also showed additional genetic alterations such as losses of *IL18, CASP6, RENT2* and gains of *LMNA, PTPN1, MFHAS1* and *CTSB* ("genetic profile").

Interestingly, ~~By~~ by MLPA we have ~~interestingly~~ observed that 2 out of 21 pairs of primary tumors and corresponding second primary tumors showed a highly similar genetic profile, which suggest that in fact they may be a recurrence or a metastasis or ~~maybe~~ a second~~f~~ field tumor, and conversely, 7 out of 15 pairs of primary tumors and corresponding recurrences showed very low genetic similarity, which could indicate that they may rather be a second primary tumor. Clinical criteria are not always useful to define HNSCC development toward second primary tumor or recurrence and it may be aided by taking into account genetic alterations.

CONCLUSIONS

Our study has shown several genetic differences and similarities between primary tumors and second primary tumors, recurrences in HNSCC, and some of them had not been previously related to tumor progression. Further investigation is necessary to confirm their possible role in the development and progression of HNSCC. Comparison of primary tumors, second primary tumors and tumor recurrences genetic profiles suggest that clinic-pathological criteria do not always accurately differentiate these entities. Genetic profiling may be useful or helpful in the diagnosis and prognosis of these difficult cases.

REFERENCES

1) Sikora AG, Toniolo P, DeLacure MD. The changing demographics of head and neck squamous cell carcinoma in the United States. Laryngoscope. 2004;114:1915-23.
2) González García R, Naval Gías L, Román Romero L, Sastre Pérez J, Rodríguez Campo FJ. Local recurrences and second primary tumors from squamous cell carcinoma of the oral cavity: a retrospective analytic study of 500 patients. Head Neck. 2009; 1168-1180.
3) Álvarez Marcos C, Llorente Pendás JL, Franco Gutiérrez V, et al. Tumour Recurrence in Squamous Head and Neck Cancer. Acta Otorrinolaringol Esp. 2007;58:156-63.
4) Brenner B, Marshak G, Sulkes A, Rakowsky E. Prognosis of patients with recurrent laryngeal carcinoma. Head Neck. 2001;23: 531-5.
5) Álvarez Marcos C, Llorente Pendás JL, Franco Gutiérrez V, et al. Second primary tumors in head and neck cancer. Acta Otorrinolaringol Esp 2006;57:462-466.
6) Dooghe IJ, De Vos M, Van Cauwenberge PB. Multiple primary tumors in patient with head and neck cancer: results of a prospective study and future perspectives. Laryngoscope. 1998, 108: 250-256.
7) Rennemo E, Zätterström U, Boysen M. Impact of Second Primary Tumors on Survival in Head and Neck Cancer: An Analysis of 2,063 Cases. Laryngoscope. 2008; 118: 1350-6.
8) Álvarez Marcos C, Llorente Pendás JL, Franco Gutiérrez V, et al. Distant metastases in head and neck cancer Acta Otorrinolaringol Esp 2006;57:369-372.
9) Braakhuis BJ, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. Head Neck. 2002; 24: 198-206.

10) Álvarez Marcos C, Alonso Guervós M, Rodríguez Prado N, et al. Genetic model of transformation and neoplastic progression in laryngeal epithelium. Head Neck 2010 (Epub ahead of print, doi:10.1002/hed.21432).

11) Cabanillas R, Llorente JL. The Stem Cell Network model: clinical implications in cancer. Eur Arch Otorhinolaryngol. 2009; 266: 161-70.

12) Wong LY, Wei WI, Lam LK, Yuen AP. Salvage of recurrent head and neck squamous cell carcinoma after primary curative surgery. Head Neck. 2003; 25: 953-9.

13) Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002; 30: e57.

14) Almadori G, Bussu F, Cadoni G, et al. Multistep laryngeal carcinogenesis helps our understanding of the field of cancerization phenomenon: a review. Eur J Cancer. 2004; 40: 2383-8.

15) Hermsen M, Alonso Guervós M, Meijer G, et al. Chromosomal changes in relation to clinical outcome in larynx and pharynx squamous cell carcinoma. Cell Oncol. 2005; 27: 191-8.

16) Tremmel SC, Götte K, Popp S, et al. Intratumoral genomic heterogeneity in advanced head and neck cancer detected by comparative genomic hybridization. Cancer Genet Cytogenet. 2003; 144: 165-74.

17) Singh B, Stoffel A, Gogineni S, et al. Amplification of the 3q26.3 locus is associated with progression to invasive cancer and is a negative prognostic factor in head and neck squamous cell carcinomas. Am J Pathol. 2002; 161: 365-71.

18) Califano J, van der Riet P, Westra W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res. 1996; 56: 2488-92.

19) Sasiadek MM, Stembalska-Kozlowska A, Smigiel R, Ramsey D, Kayademir T, Blin N. Impairment of MLH1 and CDKN2A in oncogenesis of laryngeal cancer. Br J Cancer. 2004; 90: 1594-9.

20) Smigiel R, Stembalska-Kozlowska A, Mirghomizadeh F, et al. Correlation among loss of heterozygosity, promoter methylation and protein expression of MLH1 in larynx cancer. Oncol Rep. 2004;11: 707-10.

21) Lee JW, Kim MR, Soung YH, et al. Mutational analysis of the CASP6 gene in colorectal and gastric carcinomas. APMIS. 2006; 114: 646-50.

22) Rosin MP, Cheng X, Poh C, et al. Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. Clin Cancer Res. 2000; 6: 321-2.

23) Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res. 2003; 63: 1727-30.

24) Schoop RA, Kooistra K, Baatenburg De Jong RJ, Noteborn MH. Bcl-xL inhibits p53- but not apoptin-induced apoptosis in head and neck squamous cell carcinoma cell line. Int J Cancer. 2004; 109: 38-42.

25) Capaccio P, Pruneri G, Carboni N, et al. Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes. Head Neck. 2000; 22: 234-40.

26) Nogueira CP, Dolan RW, Gooey J, et al. Inactivation of p53 and amplification of cyclin D1 correlate with clinical outcome in head and neck cancer. Laryngoscope. 1998, 108: 345-50.

27) Rodrigo JP, García LA, Ramos S, Lazo PS, Suárez C. EMS1 gene amplification correlates with poor prognosis in squamous cell carcinomas of the head and neck. Clin Cancer Res. 2000; 6: 3177-82.

28) Guervós MA, Marcos CA, Hermsen M, Nuño AS, Suárez C, Llorente JL. Deletions of N33, STK11 and TP53 are involved in the development of lymph node metastasis in larynx and pharynx carcinomas. Cell Oncol. 2007; 29: 327-34.

29) Kim MM, Califano JA. Molecular pathology of head and neck cancer. Int J Cancer 2004;112:545-553.

Figures legend

Figure 1. Percentage of genetic losses obtained by MLPA in 21 primary tumors (white bars) and their corresponding second primary tumors () and 15 primary tumors (black bars) and their corresponding tumor recurrences ().

Figure 2. Percentage of genetic gains obtained by MLPA in 21 primary tumors (white bars) and their corresponding second primary tumors () and 15 primary tumors (black bars) and their corresponding tumor recurrences ().

Figure 3. Genetic profile and genetic fingerprint of primary tumors, second primary tumors and recurrences. Only the genetic alterations higher that 33% for gains and losses and higher that 24% for amplifications are showed. (-:loss, ; +: gain; ++: amplification).

Figure 4. Genetic profile from a supraglottic primary tumor (A1) and their corresponding "low similarity" recurrence (A2) and from a glottic primary tumor (B1) and their corresponding "high similarity" second primary tumor (B2). Each bar indicated the relative copy number value of a gene analyzed by MLPA; Values lower than 0.8 were interpreted as losses, higher that 1.2 as gains and higher than 2.2 as amplifications. The genetic profile of each sample is represented by the black line.