Significant involvement of chromosome 13q deletions in progression of larynx cancer, detected by comparative genomic hybridization

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous group of tumours with various clinical characteristics. These tumours generally exhibit complex karyotypes. Few studies of genomic imbalances have been performed exclusively in subgroups of larynx cancer samples at different stages of the disease. In the present study, chromosomal gains and losses were investigated in 52 larynx tumours, by using comparative genomic hybridization (CGH). The mean number of observed alterations was 37.7 per tumour. The most common sites of losses were 1p, 13q, Xp, and the most common gains were located in 1p, 9q, 16q. The overall number of gains was negatively associated with cancer grading. G1 tumours were also characterized by a higher frequency of deletions in 13q32 and amplifications in 1q23, than tumours in other grades (p < 0.05). The frequency of losses of 13q22 also positively associated with tumour size. There was no association between the frequency of losses in 13q and the presence of lymph node metastases at the time of diagnosis. Another statistically significant association was observed for gains at 1q22-23 and tumour size (p < 0.01). No statistically significant difference in the frequency of most common imbalances was detected between primary tumours with lymph node metastases and those without metastases. In conclusion, we discovered a significant involvement of 13q deletions in the progression of larynx cancer. All the other significant changes observed in the present study were reported previously as being important for HNSCC progression. It seems that multiple genes are disrupted in the process of neoplastic transformation in the larynx, and the networks of events remain to be elucidated.

Key words: CGH, genomic imbalances, larynx cancer.
The loss of genetic material was observed most often in 3p, 5q, 7q, 8p, 9p, 11q, 13p, 14p, 15p and 18q, whereas chromosomal gains were detected mostly in 1q, 3q, 8q, 15q and 11q13 (Jin et al. 1993; Jin et al. 1995; Mertens et al. 1997). Another technique widely used in recent years to study chromosomal imbalances in cancer is comparative genomic hybridization (CGH), which enables the detection of changes at a resolution of 3–10 Mb. The results of studies on head, neck and buccal cavity tumours (premalignant lesions, primary, metastatic and cell lines) by using this technique were reviewed by Struski et al. (2002). In 494 cases summarised from 21 publications, chromosomal changes were observed most often (> 40% of cases) in 3q, 5p, 7p, 8q, 11q, 17q and 20q. A variety of oncogenes localised in those regions were hypothesised to play a role in the aetiology of HNSCC.

An earlier study on 20 pairs of larynx cancer samples (tumour specimens and metastases to adjacent lymph nodes) revealed a significant increase in frequency of 13q deletions in metastases (74% of samples) in comparison with primary tumours (42% of samples) (Kujawski et al. 2002). The relationship between the frequency of 13q deletions and the histological grade of primary tumours remains unclear. The aim of the present study was to analyse chromosomal imbalances in a set of 52 primary squamous cell carcinoma of the larynx (SCCL) and to compare the frequency of observed aberrations with regard to tumour stage (according to TNM classification) and grade.

Material and methods

Patients

The study was performed on 52 SCCL patients (46 males and 6 females). Unlike most other studies of genomic imbalances in HNSCC, the present group of patients was limited only to larynx cancer cases. None of the patients had a family history of cancer. The fresh tumour material and blood samples were obtained before any treatment at the Department of Otolaryngology, Wroclaw Medical University. The tumours were diagnosed histopathologically according to the guidelines of the World Health Organisation. Three tumours were stage I, one was stage II, twenty four were stage III and twenty four were stage IV. Sixteen patients had lymph node metastases. No patient had distant metastases at the time of diagnosis. Histological grade was well differentiated (G1) in 9 cases, moderately differentiated (G2) in 30 cases, and poorly differentiated (G3) in 13 cases. Due to the small number of patients with tumours in stages I and II, the group was divided into only two subgroups (I-III stage versus IV stage) for the purpose of statistical analysis of the correlation between the number of imbalances and tumour stage.

The experimental protocol was approved by the Ethics Committee of the Wroclaw Medical University.

Comparative genomic hybridization (CGH)

CGH was performed according to standard procedures (Kallioniemi et al. 1994). Normal male (46,XY) metaphase slides (Metaphase CGH Slides; Vysis GmbH) were treated with 1 mg mL–1 RNAse (Sigma) for 20 min and 0.001% pepsin A (Sigma) for 5 min at 37°C, denatured in 70% formamide / 2 × SSC, dehydrated in a cold ethanol series (–20°C; 70, 90, 100%) for 2 min, and air-dried. Human reference DNA was prepared from peripheral blood lymphocytes of a normal male donor. It was labelled with rhodamine-5-dUTP (R-5-dUTP, Roche) after PCR amplification through degenerate oligonucleotide primed-PCR (DOP-PCR) (Telenius et al. 1992). Tumour DNA was isolated from larynx cancer specimens and labelled with fluorescein-12-dUTP (F-12-dUTP, Roche) through DOP-PCR. DOP-PCR was performed in 25 μL of a mixture containing 100 ng of genomic DNA, 0.2 mM of each dATP, dGTP, dCTP, dTTP (Finnzymes), 0.1 mM F-12-dUTP or R-5-dUTP, 3 mM MgCl₂, 20 μM of a universal degenerate primer (5’-CCG ACT CGA NNN ATG TGG-3’) and 0.5 units of Taq polymerase (Gibco BRL). PCR amplification was done with an initial denaturation at 94°C for 1 min, followed by 8 cycles for 1 min at 94°C, 1 min at 40°C, and 4 min at 72°C. This was followed by 20 cycles for 1 min at 94°C, 1 min at 56°C, 2 min at 72°C and one additional cycle for 1 min at 94°C, 1 min at 56°C, and 5 min at 72°C. DOP-PCR products were treated with 0.01 U μL⁻¹ DNAse for 3 min at 95°C, resulting in probe size between 200 and 500 bp. Fluorescein- and rhodamine-labelled DNA were precipitated together in the presence of 10 μg human Cot I DNA and 25 μg herring-sperm DNA (Gibco BRL). The pellet was dissolved in 12 μL of hybridization solution (20% dextran sulfate, 4 × SSC, 50% formamide), denatured for 10 min at 96°C, and preannealed for 30 min
at 37°C. The probe mixture was then applied onto pretreated normal metaphase slides. Hybridization was carried out for 48 h at 37°C in a moisture chamber under a 18 mm × 18 mm cover slip sealed with rubber cement. The slides were washed at 42°C, 3 times in 50% formamide / 2 × SSC, 3 times in 2 × SSC, and 3 times in 0.1 × SSC, 5 min each time. Slides were then dehydrated in an ethanol series (70, 90, 100%) for 2 min at room temperature and air-dried. Chromosomes were counterstained with 0.3 μg mL⁻¹ DAPI in the Vectashield solution (Vector).

Image acquisition and evaluation were done using a Leica DM-RB epifluorescence microscope equipped with a Kappa CF 8/1 DX camera controlled by the ISIS software (MetaSystems GmbH). Three colour images, green for tumour DNA, red for reference DNA, and blue for the DAPI counterstain were acquired from 10–20 metaphases per sample. The threshold values for detection of genomic imbalances were: 0.8 for losses and 1.25 for gains. All centromeres, as well as the heterochromatic regions of chromosomes Y, 1, 16 and 19, were excluded from further analysis, because these regions can yield unreliable CGH data, due to incompletely suppressed repetitive DNA sequences.

**Statistical analysis**

The test for difference in proportions was used to analyse the frequency of gains and losses. Student’s t-test, Mann-Whitney test, and Fisher exact test for independence were used to analyse associations between the group of tumour samples with deletions in the 1q22-23 and 13q regions detected by CGH and the group of tumour samples without deletion in these regions. All the calculations were carried out with the STATISTICA package.

**Results**

In the present study, the CGH analysis of chromosomal imbalances was performed on 52 SCCL samples. An overview of the CGH results is provided in Figure 1. Altogether, 1961 unbalanced chromosomal changes were detected: 1042 losses and 919 gains. The mean number of alterations was 37.7 per tumour. Statistically significant changes (p < 0.05) in DNA copy number are presented in Table 1. The most common sites of losses were 1p34.1-p36.3 (33% of cases), 13q21-q32 (42%), Xp21-p22.1 (37%), and the most common gains were located in 1p13-q23 (62%), 9q13-q21 (54%) and 16q13 (37%).

![Figure 1. Summary of DNA copy number changes in 52 patients with larynx cancer. Bars on the left side of each chromosome ideogram denote losses of sequences in the tumour genome, and bars on the right side denote gains.](image-url)
We analysed associations between clinico-histopathological characteristics of tumours and the overall number of gains and losses (Table 2), as well as statistically significant gains and losses (Table 3). The overall number of gains was negatively correlated with cancer grading. G1 tumours were characterized by a higher overall number of gains and a higher frequency of deletions in 13q32 and amplifications in 1q23, than tumours in other grades (p < 0.05 and p < 0.01, respectively). Statistically significant associations (p < 0.01) were also observed between tumour size and gains at 1q22-24.3 and loss of 13q22 (Table 3). No statistically significant difference in the frequency of most common imbalances was detected between primary tumours with lymph node metastases and those without metastases.

Table 2. Associations between number of imbalances and histopathological features of cancer cells observed in 52 larynx cancer samples

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Mean no. of gains (SD)</th>
<th>Mean no. of losses (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G1</td>
<td>9</td>
<td>24.6710.98*</td>
<td>20.1112.31</td>
</tr>
<tr>
<td>G2</td>
<td>30</td>
<td>15.6010.79</td>
<td>16.5710.25</td>
</tr>
<tr>
<td>G3</td>
<td>13</td>
<td>9.549.32*</td>
<td>17.5410.68</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–T3</td>
<td>28</td>
<td>12.811.20</td>
<td>17.010.8</td>
</tr>
<tr>
<td>T4</td>
<td>24</td>
<td>19.0010.84</td>
<td>17.9610.53</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>36</td>
<td>15.1910.69</td>
<td>18.2510.58</td>
</tr>
<tr>
<td>N1–N3</td>
<td>16</td>
<td>16.6913.09</td>
<td>15.5610.71</td>
</tr>
<tr>
<td>All patients</td>
<td>52</td>
<td>15.6511.38</td>
<td>17.4210.59</td>
</tr>
</tbody>
</table>

*statistically significant difference (Student’s t-test, p < 0.05)

Table 3. Associations between the most common gains or losses and histopathological features of cancer cells observed in 52 larynx cancer samples

(a) 13q32

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumours with deletion</td>
<td>4 (33.3%)</td>
<td>7 (58.3%)</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>Tumours without deletion</td>
<td>5 (12.8%)</td>
<td>22 (56.4%)</td>
<td>12 (30.8%)</td>
</tr>
</tbody>
</table>

Association between tumour grading (G) and deletions in 13q32 (Mann-Whitney test, p < 0.05)

(b) 13q22

<table>
<thead>
<tr>
<th></th>
<th>T1-T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumours with deletion</td>
<td>4 (28.6%)</td>
<td>10 (71.4%)</td>
</tr>
<tr>
<td>Tumours without deletion</td>
<td>23 (63.9%)</td>
<td>13 (36.1%)</td>
</tr>
</tbody>
</table>

Association between tumour size (T) and deletions in 13q22 (Fisher’s exact test, p < 0.05)

(c) 1q22

<table>
<thead>
<tr>
<th></th>
<th>T1-T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumours with amplification</td>
<td>7 (31.8%)</td>
<td>15 (68.2%)</td>
</tr>
<tr>
<td>Tumours without amplification</td>
<td>18 (69.2%)</td>
<td>8 (30.8%)</td>
</tr>
</tbody>
</table>

1q23

<table>
<thead>
<tr>
<th></th>
<th>T1-T3</th>
<th>T4</th>
</tr>
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<tbody>
<tr>
<td>Tumours with amplification</td>
<td>2 (16.7%)</td>
<td>10 (83.3%)</td>
</tr>
<tr>
<td>Tumours without amplification</td>
<td>26 (65.0%)</td>
<td>14 (35.0%)</td>
</tr>
</tbody>
</table>

Association between tumour size (T) and amplifications in 1q22 and 1q23 (Fisher’s exact test, p < 0.05 and p < 0.01, respectively)

(d) 1q23

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumours with amplification</td>
<td>5 (41.7%)</td>
<td>7 (58.3%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Tumours without amplification</td>
<td>4 (10.0%)</td>
<td>23 (57.5%)</td>
<td>13 (32.5%)</td>
</tr>
</tbody>
</table>

Association between tumour grading (G) and amplification in 1q23 (Mann-Whitney test, p < 0.01)

Discussion

Tumour samples from HNSCC patients usually present a high heterogeneity of genetic imbalances. Although a few common imbalances detected by CGH and karyotype analysis were repeatedly reported, it is expected that tumours diagnosed in different sites of the head and neck could be characterized by specific patterns of chromosomal aberrations. In the present study, multiple imbalances were found. Chromosomal losses were observed more often than gains (1042
Deletion of 13q in larynx cancer

The mean copy number of chromosomal imbalances per case was 15.65 for deletions and 17.42 for gains. Singh et al. (2001) in their study on 11 HNSSCC cell lines observed a median of 16 copy number changes. Thus, HNSSCC seems to be more cytogenetically unstable than many other solid tumours, such as lung cancer (median of 13 copy number changes) and colorectal carcinoma (median of 5.6 copy number changes) (Ried et al. 1996; Petersen et al. 1997). However, cell lines derived from breast cancer are characterised by greater instability, with about 19.6 copy number changes derived from breast cancer are characterised by greater instability, with about 19.6 copy number changes per case (Kytola et al. 2000).

In the present study, high frequencies of chromosomal losses at 1p34.1-p36.3 and 13q21-q32 were observed. Deletions in 1p are often detected in many types of cancers, e.g. colon, breast, ovary, oral cavity, oropharynx cancer and neuroblastoma (Kaghad et al. 1997; di Vinci et al. 1998; Alvarez et al. 2001). Allelic loss in 1p36 was correlated with advanced histological grade in ovarian carcinoma (Alvarez et al. 2001). Deletion in 1p32-pter was also reported to be an early event in colon cancer development, important for the initiation of carcinogenesis (Bardi et al. 1993; di Vinci et al. 1998). Ogunbiyi et al. (1997) showed that allelic deletion of 1p36 in adenocarcinoma of the colon is an independent marker of unfavourable clinical outcome in this type of cancer. Putative apoptosis genes, p73, DR3 and Cdc2L1/2, are located in this chromosomal region (Lathi et al. 1995; Kaghad et al. 1997; Grenet et al. 1998; Sunahara et al. 1998).

Deletions in 13q were described in lung and head and neck cancers by several authors (Field et al. 1996; Larramendy et al. 2000; Luk et al. 2001; Gollin et al. 2001). Califano et al. (1996, 1999) suggested that deletions in chromosome 13 play an important role in genetic progression of HNSSCC. They described changes in 13q21 in benign tumours of the buccal cavity. The benign tumours can be the source of clonal premalignant cells and evolve to primary neoplasms (Califano et al. 1999). Kujawski et al. (1999, 2002) suggested that losses in 13q play a role in metastasis formation in HNSSCC. In the present study we replicated the finding of significant involvement of 13q deletions in larynx cancer. The deletions were significantly more frequent in well-differentiated tumours, as compared with G3 tumours. We did not find any correlation between the frequency of losses in 13q and the presence of lymph node metastases at the time of diagnosis, but the frequency of 13q22 deletions did correlate with tumour size. It seems that accumulation of 13q changes is associated with less aggressive types of tumours, independently of metastases formation. However, the lack of tissue material from metastases did not allow us to compare 13q changes in primary versus metastatic tumours, and such an association cannot be excluded.

Several genes located in 13q were implicated in cancer formation and progression. Retinoblastoma gene RB1 is located in 13q14, proximal to 13q21-q32. Pack et al. (1999) reported a high frequency of LOH at the RB1 locus and deletion at 13q12-q31 in oesophageal cancers. The authors suggested that the candidate tumour-suppressor gene on 13q implicated in oesophageal squamous cell carcinoma is a novel gene, distinct from the RB1 locus. The BRCAl2 gene is located in 13q12-q13. Mutations in the BRCAl2 gene were reported to predispose to various types of cancer, including larynx cancer (Easton et al. 1997). The loss of heterozygosity in the region proximal to 13q12-q13 suggested that this region contains an unknown tumour suppressor gene (Krikpatrick et al. 1997).

The overall number of gains observed in the present study was negatively correlated with cancer grading. Among regions in which the gains were observed most frequently, 1q22 and 1q23 seem to be particularly interesting, because gains in these regions are associated with tumour size (and IV clinical stage). Gains at 1q in HNSSCC were previously described and can be important for cancer progression (Speicher et al. 1995; Kujawski et al. 1999). A significant number of losses in chromosome X, detected in the present study, were also previously observed. Van Dyke et al. (1994) found a loss of the short arm of chromosome X in 70% of patients with HNSSCC.

The convergence of patterns of genomic imbalances in different types of squamous cell carcinoma suggests that shared genes are involved in tumour progression. As a variety of chromosomal alterations are associated with this tumour type, it appears that multiple genes are disrupted in the process of HNSSCC neoplastic transformation, and the networks of events remain to be elucidated.

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