

Three distinct regions of deletion on 13q in squamous cell carcinoma of the larynx

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Abstract. Genetic etiology of squamous cell carcinoma of the larynx (SCCL) is very complex, with both molecular and chromosomal alterations involved. The target genes have not yet been clearly identified. Therefore, our study focused on searching for regions that potentially harbor genes related to SCCL. After comparative genomic hybridization (CGH) analysis of a set of 52 SCCL we specified 13q21-q32 and 13q34 as the most frequently deleted regions. In order to precisely map the critical region of deletion, we studied these areas by using 15 microsatellite markers. In our material a significantly high frequency of loss of heterozygosity (LOH) (test for a difference in two proportions, $p < 0.001$) was observed for the following markers: D13S1320 (13q21.1), D13S800 (13q21.3), D13S1818 (13q32.1), D13S770 (13q32.3) and D13S285 (13q34). Three hot spots of LOH were found: 13q21.1-q22.1 (D13S1320-D13S1824-D13S800-SHGC30014-WI-16413-D13S1186), 13q.31.1-13q32.3 (D13S317-D13S1818-D13S770), 13q34 (D13S285). Among these areas, 13q31.1-q32.3 was identified as new hot spot of deletion in SCCLs.

Introduction

Intensive studies on the genetic etiology of HNSCC (head and neck squamous cell carcinoma) have shown that the etiology of HNSCC is very complex, with both molecular and chromosomal alterations involved (1). Chromosomal analyses, employing classic and molecular techniques, revealed the presence of a variety of balanced and imbalanced chromosomal aberrations in HNSCC cells. The karyotypes were shown to be complex with the breakpoints underlying chromosomal alterations located mainly at 1p, 1q, 3p, 3q, 4q, 8p, 8q, 9p, 10p,

10q, 11q, 13q, 14q and 15q. Among the recurrent structural chromosomal aberrations, isochromosomes 8q, deletion at 3p, and homogeneously staining regions at 11q13 were most often observed (2-4). The chromosomal model of HNSCC development and progression indicates two common genetic pathways: one with -1p, -1q and -7q as early events, followed by -8p and -4p, and another one starting with +7q, and subsequently followed by +11p, +8q and +1p. Both pathways then converge to a common set of imbalances: -3p, -9p and -11q (5).

Comparative genomic hybridization enabled the identification of chromosomal imbalances. Gains were observed mainly in 3q and 8q, while losses occurred in 3p, 13q and 22q (6,7). Huang *et al* proposed that +3q/-3p are the most important chromosomal events in the genetic etiology of HNSCC, which then may be followed by additional chromosomal imbalances, occurring with various frequencies depending on the tumor location (pharyngeal, laryngeal and oral squamous cell carcinoma) (8). The molecular data also revealed an involvement in the etiology of HNSCC of a variety of genes, such as oncogenes, e.g. *MYC*, *RAS*, *ERBB2*, *BCL2*, and tumor suppressor genes (TSG), e.g. *TP53*, *RB* (9-13). A sequence of frequent allelic loss observed at 9p, 3p, 17q, 4q, 13q followed by *LOH* at 18q and 8p has been suggested as a critical pathway for HNSCC development and progression (14).

However, the target genetic alterations for carcinogenesis of HNSCC have not yet been precisely specified. Therefore, our study focused on searching for the regions which potentially harbor genes critical in squamous cell carcinoma of the larynx (SCCL), which is the most frequently observed cancer among HNSCCs.

Materials and methods

We studied a homogeneous set of 52 primary squamous cell carcinoma of the larynx (70.6% transglottic SCCL-s). Cancer tissues and matched blood samples were obtained from the Department of Otolaryngology, Wrocław Medical University, Poland. Biological material for molecular analysis was collected before chemotherapy and/or radiotherapy. DNA was isolated from cancer tissue (the tumor specimens frozen) and the corresponding peripheral blood lymphocytes using standard techniques. To minimize the possible bias resulting from the contamination of tested samples by the normal tissue,

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Figure 1. Chromosome losses on 13q detected by CGH, in the set of 52 squamous cell carcinoma of larynx.

a dissection of tumor was performed to remove non-tumor tissue. In all of the samples tested more than 70% of the cells were cancer cells. The program of the study was accepted by the University Ethics Committee. None of the patients had a history of hereditary cancer.

To search for the regions which potentially harbor genes critical in SSCL, CGH and LOH were applied. In the first step CGH was performed on 52 tumors. Seventeen tumors were found to carry the 13q21-q32 (Fig. 1), 13q34 deletions ($p < 0.01$,

Table I. Results of LOH analyses in two set of squamous cell carcinoma of larynx, one presented with (cases 1-17) and the other without (cases 17-34) a deletion 13q21-q34 observed in CGH.

No.	Deletions 13q21-q32, 13q34 in CGH (bp)	D13 S1320 13q21.1 224-268	D13 S1824 13q21.1 164-188	D13 S800 13q21.3 295-319	SHGC 30014 13q22.1 100	WI- 19625 13q22.1 257	WI- 16413 13q22.1 132	D13 S755 13q22.1 205	D13 S1186 13q22.1 151	WI- 17550 13q22.1 150	D13 S317 13q31.1 175-199	D13 S1818 13q32.1 239-255	D13 S770 13q32.3 234-258	D13 S1820 13q33.3 187-202	D13 S1315 13q34 162-180	D13 S285 13q34 113
1	+	LOH	LOH	Het	LOH	H	Het	H	LOH	Het	LOH	LOH	LOH	Het	Het	LOH
2	+	LOH	Het	Het	LOH	H	Het	H	N	H	Het	LOH	LOH	Het	Het	Het
3	+	Het	H	LOH	Het	H	Het	H	N	Het	LOH	LOH	LOH	Het	H	LOH
4	+	LOH	LOH	LOH	Het	H	Het	H	LOH	H	Het	LOH	Het	Het	LOH	LOH
5	+	LOH	Het	LOH	N	H	H	H	N	Het	LOH	LOH	Het	Het	Het	Het
6	+	H	Het	Het	Het	H	Het	H	LOH	Het	Het	Het	H	Het	Het	Het
7	+	Het	Het	Het	Het	H	Het	H	Het	Het	Het	Het	Het	Het	Het	Het
8	+	Het	Het	Het	Het	H	H	LOH	LOH	Het	Het	Het	Het	Het	Het	Het
9	+	LOH	LOH	Het	Het	H	LOH	H	LOH	Het	LOH	Het	Het	Het	LOH	LOH
10	+	Het	LOH	LOH	Het	H	Het	H	LOH	Het	LOH	LOH	LOH	LOH	Het	Het
11	+	LOH	LOH	Het	LOH	H	Het	H	Het	Het	LOH	LOH	LOH	LOH	H	LOH
12	+	LOH	H	LOH	Het	H	Het	H	LOH	Het	Het	LOH	LOH	LOH	H	LOH
13	+	Het	Het	LOH	Het	H	Het	H	Het	Het	Het	Het	LOH	Het	H	LOH
14	+	LOH	LOH	LOH	LOH	H	LOH	H	LOH	Het	LOH	LOH	LOH	LOH	H	Het
15	+	Het	H	Het	LOH	H	Het	Het	Het	Het	Het	Het	LOH	Het	Het	Het
16	+	LOH	Het	LOH	LOH	H	Het	H	LOH	Het	LOH	LOH	LOH	Het	LOH	Het
17	+	LOH	LOH	LOH	LOH	H	Het	H	Het	Het	Het	LOH	LOH	Het	Het	Het
18	-	LOH	LOH	LOH	H	LOH	N	H	Het	H	H	Het	N	LOH	H	H
19	-	Het	Het	Het	H	H	LOH	H	N	H	H	H	LOH	Het	Het	H
20	-	LOH	H	LOH	H	Het	LOH	H	Het	H	Het	Het	Het	H	LOH	H
21	-	Het	Het	Het	N	H	LOH	H	N	N	Het	Het	N	Het	Het	H
22	-	H	Het	LOH	Het	Het	N	H	Het	Het	H	Het	Het	LOH	H	H
23	-	H	H	N	N	Het	N	H	Het	Het	H	H	Het	Het	H	H
24	-	Het	Het	H	LOH	H	N	H	Het	N	Het	Het	Het	Het	Het	H
25	-	H	Het	LOH	LOH	H	LOH	H	Het	N	LOH	LOH	LOH	Het	H	LOH
26	-	Het	Het	Het	H	Het	N	H	Het	N	Het	LOH	Het	Het	Het	LOH
27	-	Het	LOH	LOH	LOH	H	N	H	Het	Het	LOH	Het	Het	LOH	Het	LOH
28	-	H	LOH	LOH	Het	H	N	H	Het	Het	LOH	LOH	LOH	H	LOH	LOH
29	-	Het	Het	Het	LOH	H	H	Het	LOH	Het	Het	Het	Het	Het	Het	Het
30	-	LOH	LOH	H	Het	H	LOH	H	Het	LOH	LOH	LOH	Het	H	LOH	H
31	-	N	Het	Het	Het	H	LOH	H	Het	Het	Het	Het	Het	H	Het	H
32	-	Het	Het	Het	Het	H	LOH	H	Het	Het	Het	Het	LOH	Het	H	Het
33	-	H	Het	LOH	Het	Het	N	H	Het	LOH	H	LOH	N	Het	LOH	LOH
34	-	Het	Het	Het	Het	LOH	LOH	H	H	Het	Het	Het	Het	H	Het	Het

LOH, loss of heterozygosity; CGH, comparative genomic hybridization; Het, heterozygosity; H, homozygosity; N, non-informative.

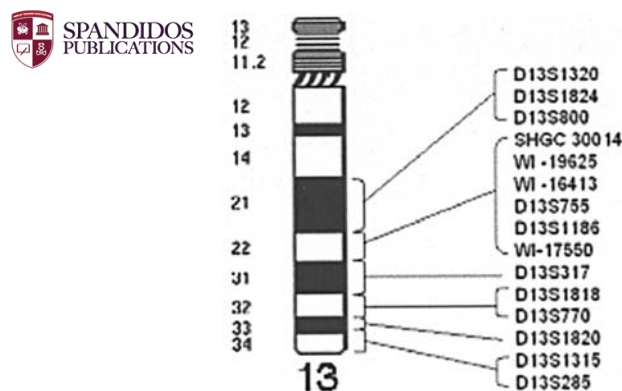


Figure 2. Localization of 15 microsatellite markers used in our study to determine LOH on 13q, in the set of 34 squamous cell carcinoma of the larynx.

test for the difference of two proportions). Therefore, LOH analysis was performed on two sets of matched tumors: 17 carrying and 17 not carrying this deletion (Table I). The LOH analyses were repeated 3 times and the results were reproducible.

CGH analysis. CGH was performed according to standard procedures (15). Briefly, human male reference DNA was labeled with rhodamine-5-dUTP (R-5-dUTP, Roche) and tumor DNA was labeled with fluorescein-12-dUTP (F-12-dUTP, Roche) using DOP-PCR. Fluorescein- and rhodamine-labeled DNA were precipitated together and hybridized to normal male (46,XY) metaphase slides (Metaphase CGH Targeted Slides; Vysis GmbH). After the hybridization, the chromosomes were counterstained with DAPI in a Vectashield solution (Vector). Image acquisition and evaluation were done using a Leica DM-RB epifluorescence microscope equipped with Kappa CF 8/1 DX camera controlled by ISIS software (MetaSystems GmbH). Three color images, green for tumor DNA, red for reference DNA and blue for DAPI counterstained were acquired from 10 to 20 metaphases per sample. The threshold values for detection of genomic imbalances were <0.8 for losses and >1.25 for gains.

LOH analysis. To determine minimal region of deletion, an analysis of loss of heterozygosity (LOH) using 15 microsatellite markers was applied (Fig. 2). The markers cover the 13q21-34 area suspected of being critical to SCCL. When screening band 13q21, the following markers were employed: D13S1320 (position 6160 kbp), D13S1824 (6505 kbp), D13S800 (7167 kbp), for band 13q22 the six following markers were applied: SHGC 30014 (7397 kbp), WI-19625 (7423 kbp), WI-16413 (7423 kbp), D13S755 (7496 kbp), D13S1186 (WI-YACMap position 187, ref. int. WC_{13.3}) and WI-17550 (7525 kbp). Four markers were used for the analysis of bands 13q31.1-13q33.3: D13S317 (8052 kbp), D13S1818 (8739 kbp), D13S770 (9732 kbp) and D13S1820 (Marshfield Map position 90.27 cM), while the telomeric region (band 13q34) was analysed using two markers: D13S1315 (10804 kbp) and D13S285 (11074 kbp) (Table I). PCR was performed according to standard protocols in a PTC-200 thermocycler (MJ Research) on DNA isolated from SCCL, as well as from matched

constitutional DNAs. Fluorescent PCR products were pooled and resolved on a 4% polyacrylamide gel supplemented with 7 M urea in an ABI-377 sequencing device. The size and quantity of fluorescent PCR products were evaluated semi-automatically by Genescan and Genotyper software using an ABI-377 sequencer (Applied Biosystems). Allelic loss was defined as more than a 70% reduction in the area of the tumor peak compared to the peak area of the corresponding normal tissue (16). In LOH analysis only informative cases (heterozygotes) were taken into account.

Statistical analysis. The frequency of LOH for markers located on 13q was analyzed. As it is accepted that LOH is associated with a cancer when the frequency of LOH is above 20% of analyzed cases, the test for difference in proportions was used to see whether any frequency was significant by greater than 20% (17-20). χ^2 test for independence, the Mann-Whitney test and Fisher's exact test for independence were used to analyze associations between the group of the tumors with deletion in region 13q in CGH and the group of the tumors without deletion in this region. All the calculations were carried out using the Statistica package.

Results

CGH analysis. In the original set of 52 LSCC 1961 chromosomal imbalances were detected. The frequency of chromosomal losses was 1042 and the frequency of gains was 919. The distribution of imbalances appeared to be non-random. In our material the highest frequency of losses was observed in chromosomal regions 13q21-q32 and 13q34 (test for a difference in two proportions compared to the overall level on all chromosomes, $p<0.01$).

LOH analysis. DNA from matched pairs of tumor and normal tissues of 34 patients were analyzed for LOH using 15 microsatellite markers. PCR was successfully performed for all markers with $>67\%$ of informative allelotypes for 13 markers (2 markers non-informative). By using informative markers, the frequency of LOH (number of cases with LOH/number of informative cases) was established as ranging from 8 to 54.6% ($39.3\pm12.8\%$; mean \pm SD). In our material a significantly high frequency of LOH (test for a difference in two proportions, $p<0.001$) was observed for following markers: D13S1320 (13q21.1), D13S800 (13q21.3), D13S1818 (13q32.1), D13S770 (13q32.3) and D13S285 (13q34). All these markers showed LOH frequency higher than 48%. A lower frequency of LOH (ranging from 38 to 43.5%), however, still significant ($p<0.01$), was detected for markers: D13S1824 (13q21.1), SHGC30014 (13q22.1), WI-16413 (13q22.1) and D13S317 (13q31.1). In the tumors bearing the deletion visible in CGH the overall frequency of LOH was higher than in the tumors not carrying this deletion (35.2 and 25.1% respectively; $p<0.05$). However, both sets of tumors presented similar pattern of LOH distribution.

The hot spots of LOH were defined as regions showing frequent LOH but retaining heterozygosity for the flanking microsatellite. Three such regions were found: 13q21.1-q22.1 (D13S1320-D13S1824-D13S800-SHGC30014-WI-16413-D13S1186), 13q.31.1-q32.3 (D13S317-D13S1818-D13S770),

13q34 (D13S285). Among these areas, 13q31.1-q32.3 was identified as a new hot spot in SCCLs.

Discussion

Our study focused on searching for regions which potentially harbor genes critical in squamous cell carcinoma of the larynx (SCCL). CGH analysis allowed us to specify two critical chromosomal regions 13q21-13q32 and 13q34 as likely targets of deletion. Our data revealing the involvement of 13q in SCCL are in good agreement with the observations of Califano *et al* and Huang *et al*, who suggested that in the HNSCC progression an inactivation of at least one and possibly several tumor suppressor genes on 13q are involved (8,14,21). The contribution of 13q to neoplastic transformation of a variety of tumors such as esophageal, head and neck, breast, ovarian and fallopian tube cancers has also been observed by many authors (13,19,20). Comparing the results of CGH analysis of tumors with and without the deletion, the pattern of LOH distribution is similar for both groups. Therefore, it can be concluded that the minimal critical region of deletion on 13q in HNSCC may not be resolved by the classic CGH approach.

Thus, to determine the hot spots of deletion within the critical regions we applied LOH analysis using 15 micro-satellite markers (positioned at distance of 400-800 kbp). This analysis confirmed and refined the presence of 3 distinct regions of deletions (hot spots): 13q21.1-q22.1, 13q.31.1-q32.3 and 13q34. High frequency of LOH in 13q14 (*RB* locus) was also observed in our material, as published elsewhere (22).

In our study 13q21.1-q22.1 was determined as a hot spot of deletion. CGH studies also revealed the involvement of this region in SCCL (23). Somatic deletion of 13q21-q22 was frequently observed in a diversity of tumors and therefore, the presence in this region of genes critical in cancerogenesis has been postulated by many authors. Up to now, key suppressor gene *BRCA2* (13q21), as well as other genes important in carcinogenesis such as protocadherin 9 (13q21-2), *KLF12* and *KLF5* (members of the mammalian Kruppel-like transcription factor family) were mapped to 13q (20,24-30). LOH in *KLF5* was observed in sporadic breast cancer, furthermore in malignant fibrous histiocytoma (MFH) and in prostate cancer (28-32).

The *CKAP2* gene (coding for the cytoskeleton-associated protein 2), which is probably important in the etiology of a variety of tumors, has been located in the region between the bands 13q14.3/13q22, while the gene *HGMW* (coding for the sciellin, a precursor to the cornified envelope of terminally differentiated keratinocytes) was found in 13q22 (33-35).

Both additional critical regions (13q.31.1-q32.3 and 13q34) were reported as frequently altered in primary nasopharyngeal carcinoma, prostate cancer and also in carcinoma hepatocellulare, HNSCC (36-41). About 20 genes were localized to 13q31-q32, some of them remaining poorly characterized. One of the most important genes located in 13q34 is *ING1* which is involved in the control of cell division and apoptosis (42). Mutations in *ING1* were found in HNSCC (43).

Concluding, ours is the first report revealing the involvement of 13q31.1-q32.3 in SCCL. Taking into account the biological functions of genes located within the delineated

region, three genes are likely to be important for HNSCC development: glypican 5 (8974-9049kbp) and glypican 6 (9157-9275 kbp), that are involved in the control of cell growth and division (13q32) and claudin 10 (9378-9392 kbp) which is an integral membrane protein and a component of tight junction strands (44-47). Further studies are necessary to verify these assumptions.

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