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LOSS OF HETEROZYGOSITY ON CHROMOSOME 13 IN SQUAMOUS CELL CARCINOMAS OF THE LARYNX*

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Objective: To locate lost region of tumor suppressor gene on chromosome 13q in squamous cell carcinoma of the larynx (LSCC) and to provide clues and evidence for discovering and locating new suppressor gene. Methods: Loss of heterozygosity (LOH) on chromosome 13q was analyzed in 58 LSCC patients by microsatellite polymorphic sequences in loci D13S765 (13q13), RB1.20 (13q14.2), D13S133 (13q14.3) and D13S318 (13q21) on chromosome 13 by PCR. Results: There weren't any LOH on chromosome 13q in 3 cases with preinvasive LSCC. Forty-five percentage (24/53) of the 53 invasive LSCC cases showed LOH at one or more loci on chromosome 13q region. The highest percentage of LOH on chromosome 13q was 52% (22/53) at D13S765 locus. Conclusion: The deletion region on chromosome 13q was located near by D13S765 locus which is centromeric to RB1. In this region there is suppressor gene, which is related to the genesis and development of LSCC, possibly including RB1. The inactivation of these suppressor genes may be related to the genesis and development of invasive LSCC.

Key words: Laryngeal neoplasms, Human chromosomes, Chromosome deletion, Tumor suppressor genes, Polymerase chain reaction.

According to Knudson's[1] "two hit" theory, detection of DNA polymorphic markers and analysis of loss of heterozygosity (LOH) in tumor cell chromosome have become one of important means to detect tumor suppressor gene inactivation and to find and locate new tumor suppressor gene.[2] To locate lost region of tumor suppressor gene on chromosome 13q in squamous cell carcinoma of the larynx (LSCC). LOH on chromosome 13q was analysed in 58 LSCC patients by microsatellite polymorphic sequences in loci D13S765 (13q13), RB1.20 (13q14.2), D13S133 (13q14.3) and D13S318 (13q21) on chromosome 13q by PCR.

MATERIALS AND METHODS

General Data

Matched normal and tumor tissue samples were obtained following surgical resection from Department of Otolaryngology of 1st Clinical College, China Medical University. The specimens were fresh frozen at -70℃. There were 3 cases of carcinoma *in situ* and 55 cases of invasive tumor. There were 28 cases of supraglottic cancer, 23 cases of glottic cancer and 3 cases of subglottic cancer. There were 5 cases of stage I, 16 cases of stage II, 16 cases of stage III and 14 cases of stage IV (according to TNM staging) in total 58 cases.

LOH Analysis

Genomic DNA of specimens were obtained

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following phenol-chloroform extraction and diluted to 100 ng/μl, reserved at 4℃. D13S765 (13q13), RB1.20 (13q14.2), D13S133 (13q14.3) and D13S318 (13q21) were chosen as DNA microsatellite poly-morphic markers to analyze LOH. RB1.20 is the 20th intron polymorphism repeated sequence of RB1 gene. PCR was performed with a total volume of 10 μl containing 100 ng genomic DNA, product labeled with α-^{32}p-dCTP was added. The PCR was subjected to 30 cycles of amplification, consisting of denaturing at 94℃ for 3 min and at 92℃ for 0.5 min, annealing at 50~55℃ for 1 min and then extension at 72℃ for 1 min. The product of PCR was diluted 2~10 times with formamide tracing fluid, denatured at 95℃ for 5 min. 1~2 μl of PCR product was separated on 5% denaturing polyacrylamide gel electrophoresis at 55℃ and 80 w for 2 h. The gels were dried in vacuum and exposed to film for 24~48 h at -70℃.

LOH Judgement

The informative cases were scored as allelic loss if the signal intensity of one allele was decreased at least in 50% in tumor DNA as compared to the corresponding allele in normal DNA.

RESULTS

There weren't any LOH on chromosome 13q in 3 cases with preinvasive LSCC on 4 loci. In 53 informative cases of 55 invasive LSCC, 24 cases showed LOH at one or more loci on chromosome 13q region. The frequency of LOH at different loci was shown in Table 1. LOH analysis at 4 loci was the same for most cases, but 4 cases showed differently as Figure 1. D13S318 loci showed heterozygocyte state. In contrast, D13S765 displayed LOH, of them, RB1.20 displayed LOH in 2 cases. Therefore, lost region on chromosome 13q in LSCC lay close to the D13S765 (13q13) locus. The frequency of LOH of different pathological differentiation was shown in Table 2. In cases of clinical stages I~II and III~IV, frequency of LOH was 36% and 51%, respectively, 3 nonsmoking nondrinking cases displayed no LOH, while the frequency of LOH in 44 smoking and drinking cases was 48% (21/44).

Table 1. LOH frequency at different loci of chromosome 13 in LSCC

Loci	Informative cases	LOH (+)	LOH (-)	LOH (%)
D13S765	42	22	20	52
RB1.20	45	18	27	40
D13S133	29	10	19	34
D13S318	39	13	26	33

	LC1	LC2	LC3	LC4
D13S765	●	●	●	●
RB1.20	○	●	△	●
D13S133	▲	●	○	○
D13S318	△	△	△	△

Fig. 1. Specimen of laryngeal cancer
● LOH △no LOH ○ uninformative
▲no amplification LC: laryngeal cancer

Table 2. LOH frequency of chromosome 13 according to pathology

Differentiation	Informative cases	LOH (+)	LOH (-)	LOH (%)
Well	17	10	7	59
Middle	23	11	12	48
Poor	5	2	3	4

DISCUSSION

Deletion on chromosome 13q is common in many tumors. Scholnick[3] studied LOH on chromosome 13 in LSCC and found that frequency of LOH at RB locus is about 50%, which suggested that the tumor suppressor gene related to development and genesis of LSCC may exist on chromosome 13q. But the deletion area in chromosome 13q is unclear. It can be determined by LOH detection in multiple loci. So we chose 4 microsatellite polymorphic markers on chromosome 13q. The results showed that the highest frequency of LOH at D13S765 is 52%, the lowest at D13S318 is 33%. The frequency of LOH at RB1.20 is 40%, that at D13S133 is 34%. In 4 cases D13S318 showed no LOH, but D13S765 showed LOH, and

LOH was found at RB1.20 locus in 2 cases. Therefore, the deletion area in chromosome 13q in LSCC lay close to the D13S765 (13q13) loci, centromeric to RB1 gene, where tumor suppressor gene exited and related to progression of LSCC.

We analysed LOH on chromosome 13q relating to carcinoma *in situ*, invasive cancer and smoking, drinking cases. The results showed that no LOH was found in 3 cases of carcinoma *in situ* and nonsmoking nondrinking cases and that LOH in invasive cancer and smoking, drinking cases was 45% and 48% respectively, which displayed that deletion of 13q is related to invasive cancer and smoking and drinking. On the other side, LOH frequency in poor differentiation cancer is lower than that of the other two type. This may show the nature of poor and well differentiation and may be caused by mutation of other genes which needs further studies.

According to Knudson's[1] "tow hit" theory, when an allelic gene deletion accompanied the mutation of the remaining loci, it is called inactivation of the gene. To find the evidence of RB gene inactivation in head and neck neoplasms, Yoo[4] determined that the minimal deletion area on chromosome 13q is between D13S118 (13q14.1–14.2) and D13S133 (13q14.3) and that the frequency of LOH was 52%. Yoo[4] also detected the expression of p110 RB1 protein, and found that only 19% of cases displayed loss of p110 RB1 protein expression, of them RB1 loci showed LOH in 13% of cases. So RB1 gene inactivation only occurred in small number, therefore Yoo[4] considered that there was a tumor suppressor gene between D13S118 and D13S133, which might be near D13S133, the distal part of RB1 gene.

Recent studies showed that there were two tumor suppressor genes DBM[5] and BRCA2[6] near the chromosome 13q14.2, (RB1 gene). DBM gene located in D13S25 (13q14.3), the distal part of RB1 gene, at least 530 kb to RB1 gene and deleted steadily in chronic B lymphocyte leukemia. BRCA2 gene was near chromosome 13q12–q13 area, centromeric to RB1 gene, being one of the suspective gene of familial breast cancer. We had no evidence to support that the DBM gene is the deletion gene in chromosome 13q in head and neck neoplasms.

In our study the deletion area of chromosome 13q located near D13S765, whose frequency of deletion was 52%. The result suggested that the tumor suppressor gene of the deletion area of chromosome 13q lay close to D13S765, centromeric to RB1 gene. At D13S765 locus LOH was found in 1 case without LOH at RB1.20, but this can't exclude no RB1 gene deletion because we chose the 20th intron polymorphic sequence in RB1 gene. If part of RB1 gene deleted, it might display LOH without RB1.20. So the tumor suppressor gene in chromosome 13q deletion area in LSCC is near D13S765, probably including RB1 gene. Further studies are needed to verify whether the target gene in chromosome 13q deletion area in LSCC includes BRCA2, such as screening mutation and deletion of RB1, DBM, BRCA2, specifically locating D13S765, etc. Our study gives a clue and evidence for further mapping of tumor suppressor gene in chromosome 13q in LSCC.

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