

A combined strategy of conventional cytogenetics, fluorescent *in situ* hybridization and microsatellite polymerase chain reaction to analyze the deletion of chromosome 6 in laryngeal squamous cell carcinoma

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Abstract. Laryngeal squamous cell carcinoma (LSCC) is a common cancer of the upper respiratory tract. The cytogenetic and molecular events involved in the pathogenesis of LSCC are not well understood. In this study, a combined strategy of conventional cytogenetics, fluorescent *in situ* hybridization (FISH) and microsatellite polymerase chain reaction was performed to analyze the deletion of chromosome 6 in LSCC. Karyotype analysis indicated that the deletions of 6q were marker chromosomes potentially specific to LSCC. To further characterize the loss of 6q, metaphase cells derived from both solid tumors and the Hep-2 cell line were investigated using FISH, with results consistent with those of conventional cytogenetics. Moreover, tumor-adjacent normal tissue DNA pairs from 70 LSCC in China were analyzed for loss of heterozygosity (LOH) and microsatellite instability (MI) on chromosome 6q in the region 6q25 by polymerase chain reaction (PCR) and three microsatellite markers. Overall, the highest frequency of microsatellite alteration (68.2%) was located at D6S980, which revealed that the region around D6S980 in 6q25 might harbor some important genes related to the pathogenesis of LSCC in Chinese patients. The pattern of chromosomal changes detected using the combined strategy suggests that it will become the most suitable way to find novel cancer-related genes and discover the relationship between the aberration of genetics and their tumorigenic mechanism.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common cancer worldwide, with more than 500,000 new cases diagnosed each year (1). Laryngeal squamous cell carcinoma (LSCC) is the second most common cancer of the head and neck region. Epidemiological studies reveal that incidence rates vary among different countries and nations (2). LSCC accounts for 1-8.4% of human cancers in China. A relatively high incidence of LSCC is found in Northeast China, possibly due to prolonged cold weather stimulation, heavy alcohol intake and tobacco use resulting in carcinogenesis (3). The curative effect and prognosis of the required operation are relatively poor and are generally followed by severe dysfunction and recrudescence. The identification of a genetic mechanism for LSCC has broad implications for understanding and preventing its occurrence. However, the cytogenetic and molecular events involved in its pathogenesis are not well understood.

Specific chromosome aberrations have been identified in some neoplastic diseases. The most remarkable progress has been achieved in hematological cancers, in which specific chromosomal abnormalities often correlate with tumor pathogenesis and play an important role in diagnosis and prognosis (4-6). Solid tumors represent a huge variety of numerical and structural chromosome aberrations. Knowledge concerning them, however, is not very advanced (7). Until now, only a few aberrations specific to a given type of tumor have been identified. Examples include t(11;22)(q24;q12) in Ewing's tumor and del(11)(p13) in Wilms' tumor (8). Instead of looking for characteristic chromosome aberrations, investigations have been focused on the most commonly occurring or typical chromosome alterations. Further studies gained momentum after Califano *et al* (9) reported a hypothetical model of the dynamics of the progression of head and neck cancer. Because of the technical difficulties encountered in solid tumor cytogenetics, including insufficient metaphases and suboptimal quality of harvesting and banding, occasions still arise in which analysis is severely limited by single conventional cytogenetics. Incomplete

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conclusions are often drawn as to the precise nature of the chromosomal abnormality, if indeed any can be detected. Mark *et al* (10) advocated adopting a rational approach of analysis of the available metaphases in tumors by a combined strategy. A significant improvement in success rates might be more readily achieved through the cytogenetic analysis of solid tumors. A der(22)t(20;22), resulting in an amplification of the proximal region of the EWS gene, was identified in a case of Ewing sarcoma using a multimodal strategy of histology, immunohistochemistry, molecular genetic and molecular cytogenetic approaches (11). A complex karyotype in primary synovial sarcoma of the heart was analyzed by cytogenetic and molecular genetic methods (12). These cases show the importance of combined approaches in identifying uncommon aberrations in solid tumors.

Regarding LSCC, numerical and structural chromosome aberrations, in particular the deletion of certain areas and bands of some chromosomes, are known to be characteristic changes (7,13). Until now, however, little attention has been focused on the relationship between chromosome 6 aberrations and carcinogenesis in LSCC. In this study, we combined conventional karyotype analysis and the fluorescent *in situ* hybridization (FISH) technique to confirm the characteristic chromosome 6 deletion in LSCC. On the basis of the cytogenetic data, we performed microsatellite polymerase chain reaction (PCR) to further detect the common deletion regions at the molecular level, with the aim of contributing to the understanding of LSCCs genetic mechanism. In short, the goal of the study was to identify the typical genetic aberrations of LSCC and, simultaneously, to illustrate the importance of the application of our combined strategy.

Materials and methods

Materials. The Hep-2 cell line, derived from ATCC, was purchased from the Beijing E.N.T. Research Institute. The 90 LSCC tissues were obtained from patients treated at the E.N.T. Department of the 463 Hospital of Air Force after obtaining their informed consent and the approval of the hospital authorities. All subjects were unrelated ethnic Chinese from Northeastern China. None had received radiotherapy or chemotherapy prior to the genetic analysis. The clinical pathological characteristics of the patients are shown in Table I according to the International Union Against Cancer. Twenty freshly operated specimens were analyzed with an improved primary cell culture. The other 70 specimens, which included cancerous tissues and paired adjacent normal laryngeal tissues (PANLs) typically 4-15 mm in diameter, were frozen immediately after collection and stored at 80°C for loss of heterozygosity (LOH) and microsatellite instability (MI). Normal cells presenting as contaminants in the primary laryngeal lesions were removed using a microdissection procedure (14). Samples containing <60% tumor cells were not taken for analysis.

Fresh LSCC samples analyzed by improved primary cell culture for chromosome preparation. Samples were processed by an improved primary cell culture for chromosome preparation. In detail, the fresh samples were minced, disaggregated in RPMI-1640 supplemented with 1 mg/ml collagenase

Table I. Clinicopathologic features of 90 patients with LSCC.

Clinicopathological parameters	Patient no.	Median age (years)	Mean age \pm SD (years)
Primary site			
Supraglottis	44	58	60.16 \pm 9.05
Glottis	31	57	60.08 \pm 8.99
Subglottis	15	53	55.56 \pm 6.86
TNM stage			
I	19	57	57.60 \pm 7.76
II	32	60	61.82 \pm 8.79
III	24	53	54.95 \pm 8.97
IV	15	63	62.85 \pm 6.78
Gender			
Male	63	57	59.47 \pm 9.33
Female	27	56	57.12 \pm 7.19
Tumor differentiation			
Well	26	58	58.65 \pm 10.14
Moderate	38	56	61.17 \pm 7.33
Poor	22	60	57.75 \pm 10.16
CIS	4	54	59.00 \pm 6.98

TNM, tumor node metastasis; CIS, cancer *in situ*.

(510 U/mg, Sigma), 8% fetal calf serum, 100 IU/ml penicillin, 0.2 mg/ml streptomycin and 2.5 μ g/ml amphotericin B at 37°C for 16 h, and plated on a gelatin (3%)-coated culture capsule in RPMI-1640 supplemented with 8% fetal calf serum, antibiotics, 2 U/ml insulin and 0.23 mg/ml glutamine. Cells were attached and grown gradually, and cultures were harvested after 20 days. Over these days, we supplemented with a medium in which the Hep-2 cell line had already been cultured for 16 h. Before using the medium, it was necessary to filter it to eliminate Hep-2 cells and avoid contamination. The *in situ* preparations were harvested in the standard manner described by Pandis *et al* (15), with minor modifications including incubating the cell suspension twice with hypotonic solution, 0.4% KCl and 0.4% sodium citrate (1:1) at 37°C for 10 min each. Metaphase cells were G-banded with Wright stain. Clonality criteria and the description of chromosome abnormalities followed ISCN 2005 (16).

High-resolution chromosome preparation for the Hep-2 cell line. Chromosome analysis of the Hep-2 cell line was performed on cells from passages 7, 11 and 15. Samples were processed by a high resolution banding technique, described previously (17). Clonality criteria and the description of chromosome abnormalities were as described above.

FISH for chromosome 6 in primary LSCC cells and the Hep-2 cell line. A chromosome 6 painting probe (Cat.No.1836366) was obtained from Boehringer Mannheim (Germany). The conditions for hybridization and post-hybridization washes were as recommended by the manufacturer.

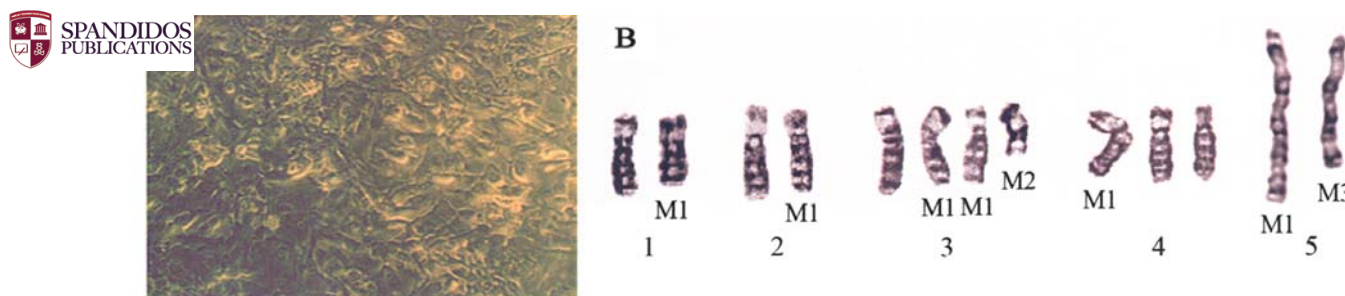


Figure 1. Conventional cytogenetic results. (A) An improved primary cell culture from a tumor (case 3) showing the shape of tumor cells (x400). (B) Karyotype of chromosomes 6 from the 4 LSCCs and the Hep-2 cell line showing, in each of them, del (6). 1, 2, 3 and 4, G-banding karyotype of the 4 LSCCs showing 6q-; 5, High-resolution G-banding karyotype of the Hep-2 cell line showing 6q-; M₁, M₂ and M₃, marker chromosome showing the deletion of 6q25-qter, 6q14-qter and 6q22-qter, respectively.

Table II. Clinicopathologic and cytogenetic data for 4 LSCCs which succeeded in improved primary cell culture.

Case no.	Age (years/sex)	Site	TNM stage	Differentiation	No. of cells having 6q-/analyzed	Diagnosis of chromosome 6 deletion
1	48/M	Supraglottis	T ₂ N ₀ M ₀	MD	7/11	del(6)(q25)
2	46/M	Glottis	T ₁ N ₀ M ₀	MD	6/10	del(6)(q25)
3	49/M	Supraglottis	T ₄ N ₀ M ₀	PD	17/22	del(6)(q25); del(6)(q14)
4	67/F	Supraglottis	T ₁ N ₁ M ₀	PD	15/20	del(6)(q25)

LOH and MI in chromosome 6q25 of LSCC. High molecular weight genomic DNA was extracted from LSCC tissues and PANLs according to standard procedure (14). Three highly polymorphic microsatellite markers were used. The markers were D6S1708, D6S437 and D6S980 at the locus of the 5'-end of *IGF2R/M6P* (insulin-like growth factor II receptor/mannose 6-phosphate) at 6q25.2, 6q25.2, and 6q25.3, respectively. Primers included D6S980 (forward primer, AGGGAGCCGA GATTGCAC, reverse primer, CTGAAGGGTGAGGAGTT TCT), D6S1708 (forward primer, TGCAATTCCAATGGGG, reverse primer, GTGGCAGAATCTCCAAGG) and D6S437 (forward primer, TGTCTGGTGGAGGCA, reverse primer, GGTACAGTGTGTTGACCCTAAGA). Information and primer sequences for the microsatellite markers were obtained from the GDB Human Genome Database (<http://www.gdb.org>). Standard PCR analysis (18) was performed in a 40 μ l reaction volume. The samples were amplified through 32 cycles in a thermal cycler comprising a 5 min predenaturing step at 95°C, cycles of 30 sec at 95°C, 45 sec at the appropriate annealing temperature (56-62°C), and 1 min at 72°C, and a final extension at 72°C for 5 min. The PCR products were concentrated by refrigerated centrifugal concentrators then diluted with a stop solution containing 98% formamide (Gibco-BRL, USA), 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturing for 10 min at 95°C, the PCR products were electrophoresed in 6% denaturing polyacrylamide gels containing 7 M urea at 600 V for 2.5-3 h. The gels were stained with silver prior to the analysis of the results. LOH was scored when the signal intensity of one allele decreased by $\geq 50\%$ compared to the corresponding normal

controls. MI was defined as the appearance of ≥ 1 new alleles in the tumor DNA as compared to its normal counterparts (19). Each assay was performed twice in order to ensure experimental reproducibility.

The χ^2 test was performed to determine the association between the tumors genetic profile (LOH/MI) and different tumor stages. $P < 0.05$ was considered statistically significant.

Results

Conventional cytogenetic (G-banded). Twenty fresh LSCC samples were analyzed by improved primary cell culture. Four cases succeeded using this method, with tumor cells growing and proliferating as distinctly shown in Fig. 1A. Quality metaphases were obtained in the four samples, which were characterized by complex karyotypes (data not shown). At chromosomes 3, 5, 6 and 17, structural aberrations were frequently seen and mostly included deletions, translocations, and isochromosomes. All four cases had del (6) as a clonal chromosome abnormality within their complex karyotype (Table II). A total of 63 metaphases in the four LSCC samples were analyzed, with 6q deletion present in 45 cells (71.4%) and most deleted regions being at 6q25-qter. Deletion of 6q was also found in 80 cells (75%) of 107 high-resolution karyotypes of the Hep-2 cell, with the breakpoint clustered at 6q25.2 and 6q22. Fig. 1B shows a representative karyotype with the deletion of chromosome 6.

Molecular cytogenetic (FISH). FISH was performed to confirm the G-banded findings, further characterize the deletion of

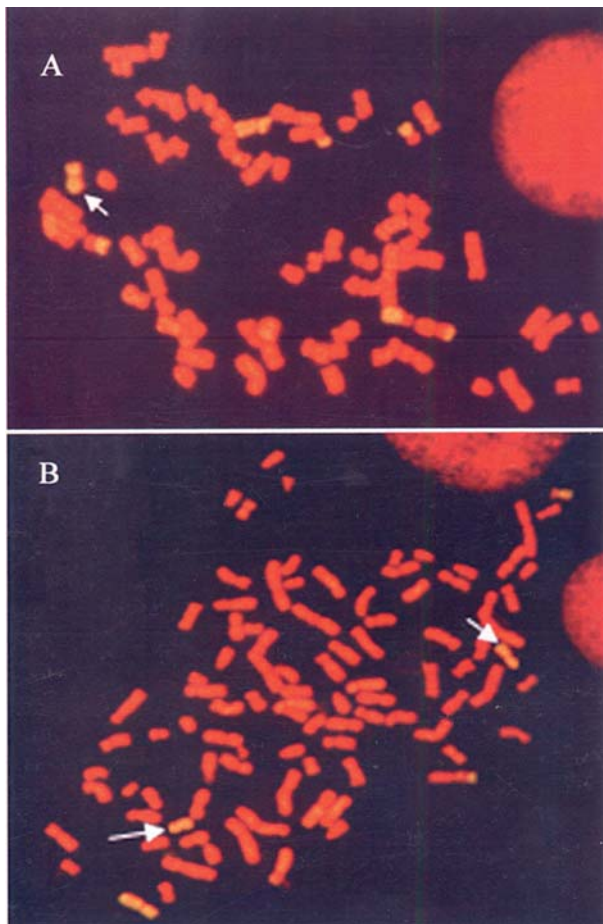


Figure 2. FISH analyses of (A) Case 2 and (B) the Hep-2 cell line using a human chromosome 6 painting probe. All deletions of chromosome 6 are indicated by arrows.

Case No	D6S 1708	D6S 437	D6S 980	Case No	D6S 1708	D6S 437	D6S 980
1				36			
2				37			
3				38			
4				39			
5				40			
6				41			
7				42			
8				43			
9				44			
10				45			
11				46			
12				47			
13				48			
14				49			
15				50			
16				51			
17				52			
18				53			
19				54			
20				55			
21				56			
22				57			
23				58			
24				59			
25				60			
26				61			
27				62			
28				63			
29				64			
30				65			
31				66			
32				67			
33				68			
34				69			
35				70			

Figure 3. Allele status of the chromosome 6q25 markers in the 70 LSCC samples. Filled squares, LOH; cross-hatched squares, MI; horizontally lined squares, retention of heterozygosity; blank squares, non-informative.

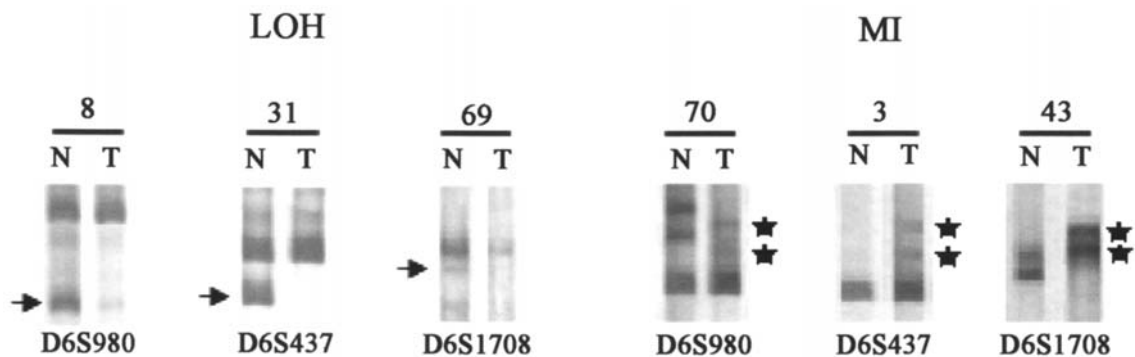


Figure 4. Representative examples of LOH and MI at various loci of chromosome 6q25. N, DNA of normal tissue; T, DNA of corresponding tumor tissue. Sample numbers are the same as in Fig. 3. Arrow, loss of the corresponding allele; asterisk, size alteration of alleles.

chromosome 6 and provide an additional estimate of the proportion of 6q- abnormal cells. A total of 141 nuclei were examined and scored for their percentage of 6q deletion. Examination of a total of 47 cells of the primary LSCCs revealed that 34 (72.3% of total) metaphase cells were 6q-. In the Hep-2 cell line, 70 (74.5%) of 94 scored cells also exhibited the deletion of chromosome 6 (Fig. 2). FISH results were therefore consistent with results obtained from the G-banded study.

Molecular genetic (microsatellite PCR). In 70 LSCC, we analyzed three polymorphic microsatellite markers for chromosomal 6q25 (Fig. 3). Representative examples of LOH and MI at various loci are shown in Fig. 4. In 64 (91.4%) of 70 patients, at least one marker was capable of giving information. Of these informative cases, 23 of 64 (35.9%) showed LOH in ≥ 1 loci. LOH was found in 10.6% (6/57), 33.3% (11/33) and 41.4% (17/41) of informative samples at D6S1708, D6S437, and D6S980, respectively. If a percentage of LOH at a locus

Clinical stages	No. of cases	LOH	χ^2 value	MI	χ^2 value
Stage I-II	40	13		8	
Stage III-IV	30	10	0.0337	6	0.0911
Total	70	23		14	
P>0.05, χ^2 test.					

was >30%, we determined that the LOH frequency of the locus was significantly high. Overall, the highest frequency (41.4%) of LOH was observed at D6S980. MI was found in 16 of 64 cases (25%) at ≥ 1 loci. Of three loci, D6S437 (5/33, 15.1%) and D6S980 (11/41, 26.8%) showed a percentage of MI >10%. The overall highest frequency (26.8%) of MI was seen in the D6S980 locus. The location of the highest LOH and MI were the same at D6S980, where the highest frequencies (68.2%) of microsatellite alterations were observed.

The association between LOH/MI at each locus and the tumor stages of LSCC patients was analyzed. There were no significant differences between LOH/MI and clinical stages (P>0.05) (Table III).

Discussion

In the present study, we used conventional cytogenetics to find that the loss of chromosome segments 6q25-qter was the most frequent structural abnormality in both primary LSCC and the Hep-2 cell line. This was further confirmed by FISH. The finding of del (6q) in each of the four LSCC cases and in the Hep-2 cell line cannot be dismissed as coincidental. It seems clear that this deletion is the karyotypic characteristic of at least a subgroup of LSCC. Because of the general technical difficulties encountered in solid tumor cytogenetics and in the analysis of LSCC in particular, the published data on LSCC cytogenetics were limited until now (13,20-22), including studies by Jin *et al* (13), Jin *et al* (20,21) and Allegra *et al* (22). We found that our results were in line only with those of Jin *et al*, who also found a loss of chromosome 6q (13), but our results regarding the frequency of its deletion were significantly higher. Because of the lack of significant difference between overall karyotypic profile and histopathologic differentiation or anatomic site (13), it can be concluded that this lack of consistent cytogenetic features in LSCC studies might be due to differences in etiology and ethnicity. Using comparative genomic hybridization analysis, we have also documented loss at chromosome 6q in LSCC in our previous studies (23). Han *et al* used a direct culture method to analyze surgical resections of LSCC from eight Chinese patients, and the loss of material from 6q was originally described as a specific change in LSCC (24). Therefore, the deletion of 6q was a prominent feature, suggesting that the gene loss engendered was relevant to the pathogenesis of LSCC and/or its progression in Chinese patients.

In addition, the deletion of 6q had been detected in a large number (>50%) of HNSCCs (25-26). The same abnormality had been repeatedly reported in a number of other malignancies including prostate cancer (27), breast cancer (28), ovarian carcinoma (29) and melanoma (30). It is evident that the loss of 6q is a common tumorigenic event in the development of many malignancies.

Tumors arise by multi-step carcinogenesis pathways involving selective losses and gains of chromosome material. Previous studies using microsatellite PCR markers to map genetic changes had mostly focused on predefined regions of the genome, the location of important genes, or chromosomes implicated in cytogenetic analysis. Our data on conventional cytogenetics and molecular cytogenetic studies in LSCC showed the most frequent deletion of 6q with breakpoints clustering at 6q25, which should be valuable for further molecular studies. The chromosomal region 6q25 may harbor some critical genes important to tumorigenesis and progression in LSCC.

In this regard, it is interesting that *IGF2R/M6P* is located in the chromosome 6q25 region. IGF2R, which carries out multiple regulatory and transport functions, plays a critical role in regulating cell growth by facilitating the activation of the growth inhibitor transforming growth factor β (TGF β), and by inactivating the growth stimulator insulin-like growth factor II (IGF2). Disruption of IGF2R function has been implicated as a mechanism which increases cell proliferation, suggesting that this receptor may play the role of a growth inhibitor (31). LOH and mutations in the remaining allele at the *IGF2R/M6P* locus have been observed in hepatocellular carcinomas (32,33), breast tumors (34), ovarian cancer (35), prostate cancer (36) and squamous cell carcinoma of the lung (37). In addition, *IGF2R/M6P* has been observed in colorectal, gastric and endometrial tumors exhibiting MI (38,39). Collectively, these studies support the hypothesis that *IGF2R/M6P* functions as a tumor suppressor gene. To the best of our knowledge, however, a relationship between LSCC and *IGF2R/M6P* had not been reported.

In this study, we used three polymorphic markers in the 3 Mb region of 6q25 to initially screen for *IGF2R/M6P* heterozygosity in 70 LSCC patients (Fig. 3). Of the 70, 64 (91.4%) were informative (heterozygous) for these polymorphisms. LOH was found in 10.6 (6/57), 33.3 (11/33), and 41.4% (17/41) of informative samples at D6S1708, D6S437 and D6S980, respectively. D6S980, located at the 5' end of *IGF2R/M6P*, had both high LOH frequency (41.4%) and high MI frequency (26.8%), which supports the hypothesis that *IGF2R/M6P* functions as a tumor-suppressor gene whose inactivation might play an important role in LSCC tumorigenesis. LOH frequency was not significantly different (P>0.05) between LOH/MI and clinical stages (Table III). Thus, *IGF2R/M6P* allele loss was a common event in LSCC irrespective of the patients clinical stage. The molecular information derived from the present study, in particular the characteristic chromosome deletion at 6q, should be valuable in further molecular studies to confirm the above hypothesis.

Moreover, to identify and characterize LSCC-related novel genes located on chromosome 6q25, our group used electric hybridization to clone a novel gene named *MTLC* (c-Myc target from laryngeal cancer cells) successfully (40). *MTLC*

was down-regulated in LSCC tissues, and could inhibit growth and promote apoptosis of laryngeal carcinoma cell line Hep-2 cells, which suggests that *MTLC* might take part in laryngeal carcinogenesis (41). These findings are in agreement with the notion that the essential methods of oncology studies are based on tumor cytogenetics, and that more attention should be paid to the frequent chromosome aberration regions where the genes specific to cancer may reside (42). These specific gains and losses of genomic material have opened the way to further detection of oncogenes, tumor suppressor genes (TSG) and the other associated genes involved in tumorigenesis.

A thorough comparison of chromosome aberrations in tumor samples and cultured material has proven that the karyotypes of cultured cells accurately reflect changes in the original material (43). Instead of the short-term culture used in most previous studies, an improved primary cell culture was adopted in the present one. The effects were accounted for by the low serum content and adaptive medium. In contrast to conventional medium with a high serum concentration (15-20%), our medium had a low serum concentration (8%), which facilitated tumor epithelial growth while inhibiting fibroblasts. LSCCs are more difficult to culture than other HNSCCs. In particular, cells with large dark nuclei and irregular forms, which most likely represent tumor cells, are extremely difficult to maintain in culture. On the other hand, fibroblast cells grow better *in vitro* and can be maintained in culture for longer times, which allows stromal fibroblasts to overgrow the tumor parenchyma cells and leads to unsuccessful tumor primary cultures. A low serum content inhibits fibroblast cells but has little effect on the proliferation of tumor parenchyma cells. Moreover, in order to encourage tumor cells to attach and grow, we supplemented the adaptive medium in which the Hep-2 cell line had already been cultured for almost 16 h. We found that this kind of medium promoted the differentiation and proliferation of tumor cells and simultaneously prevented tumor cells from detaching from the culture surface. Perhaps Hep-2 cells produce and release some of the growth factors necessary for the growth of tumor epithelial cells. With this improved medium, we were able not only to elevate the survival rate of the LSCC tumor cells, but also to obtain sufficient metaphase chromosomes to characterize LSCC in more detail.

Based on the above-mentioned data, it can be concluded that the deletion of chromosome 6q25-qter may be associated with the carcinogenesis of LSCC in Chinese patients, and that the occurrence of LOH and MI in 6q25 plays a role in its pathogenesis and progression. The commonly deleted region could harbor candidate TSGs and tumor-related oncogenes associated with the LSCC tumor. On the other hand, conventional cytogenetics via G-banding is a powerful technique which remains a gold standard despite its limitations. FISH is a useful and often necessary adjunct that has served to bridge the gap between molecular genetics and conventional cytogenetics. Molecular technologies are also useful methods by which more information may be obtained in detail. Because unsound conclusions may be drawn from cancer genetics analysis, a multimodal approach utilizing all available tools should be adopted to optimize the final outcome and give us more valuable data for further study.

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