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HEAD AND NECK

The role of genetic susceptibility in head and neck squamous cell carcinoma

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Abstract Our research is an additional genetic study to uncover the molecular mechanisms involved in head and neck squamous cell carcinoma (HNSCC) pathogenesis by studying loss of heterozygosity (LOH) and microsatellite instability (MSI) in both premalignant and malignant patients and to highlight the genotype of HNSCC in Upper Egypt. Patients with HNSCC from various parts of the world may have unique genotypes and this is the first genetic study of HNSCC in Sohag 500 KM to the south of Cairo. We performed a prospective study of 41 patients with precancerous and 79 patients with cancerous laryngeal, esophageal, nasopharyngeal, nasal and oral lesions, and 50 controls (The control patients were cases admitted for ear surgery or simple nasal surgery, from whom we took biopsy from mucosal lining of nasopharynx). The present study included 170 individuals who were admitted to the Ear, Nose and Throat department, Sohag University Hospital, Sohag, in Egypt in the period between April 2001 and March 2003. Samples which were taken by punch biopsy were frozen and stored at −80°C and were subjected to histopathological examination. We investigated LOH and MSI by using six microsatellite markers located at chromosomes 3, 5, 9, and 17. The markers used were D3S1286, D9S171, D9S753, D17S654, D17S695, and CFS1-R. LOH was in all premalignant and malignant lesions at 5q33.3-q34 and 13% of Controls. LOH at 17p21 was absent in all premalignant lesions and was found in 53% of malignant lesions and 12.4% of Controls. In premalignant lesions, LOH was at 3pter-3p24.2 (73% of cases), at 9p21 (46%), at 9q21.1-22.3 (37%), and at 17p13 (37%). These percents increased in malignant lesions to 87, 80, 67, and 63%, respectively. They were 14, 19.4, 17, and 19% in controls. Examination of LOH could improve diagnosis, adds additional confidence, in HNSCC by DNA extraction from suspicious lesions in high-risk groups (smokers and alcoholics) and LOH at 3p/9p seems to be of particular value for early detection and definition of progression risk. If there are high percent of LOH at these chromosomes, active intervention should be done (chemoprevention and regular follow up head and neck examination for very early detection and management).

Keywords Head and neck squamous cell carcinoma · Genes · Microsatellites (STRs) · Genetic instability · Loss of heterozygosity

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Introduction

Head and neck cancer, mainly squamous cell carcinoma (SCC) of the oral cavity, pharynx, and larynx, is a common human malignancy. It is the sixth most prevalent cancer in the world with a global yearly incidence of 500,000 [1] and it is an aggressive malignant neoplasm. Therefore, prevention and early diagnosis of high-risk premalignant lesions

are high priorities for reducing deaths due to head and neck cancer [2].

In the past, tumor-associated markers such as proteins/glycoproteins have been used for diagnosis or prognosis of progression in patients. However, the specificity of these assays is limited because the majority of these markers is not tumor-specific and is found in normal cells. To date, tumor-specific genetic markers have been assessed primarily in tumor biopsies. The actual sequence of the genetic alterations during head and neck SCC (HNSCC) development and progression has not yet been defined [3].

Advances in the understanding of the molecular basis of head and neck cancer should help in the identification of new markers that could be used for the diagnosis, prognosis, staging, and treatment of the disease.

Different detection methods have been used, such as microsatellite typing to show the presence of loss of heterozygosity (LOH) or microsatellite instability (MSI) and specific mutation analysis (p53 or Ki-ras), and more recent methods have used detection of aberrant methylation of the p16 gene promoter [4–6]. These specific genetic events can be detected in the saliva and serum of patients with malignancy [7] opening the door to new strategies for early cancer detection and tumor surveillance.

Loss of heterozygosity studies using microsatellite markers on head and neck malignancies have demonstrated extensive areas of loss [8]. The microsatellite markers are Short tandem repeats (STRs) which have become one of the most widely used genomic markers for identity testing and gene mapping due to their high degree of heterozygosity. Ideal STR polymorphisms amplify well under standard conditions, have a low amplification background, are easy to score and are highly informative Moreover, premalignant lesions studied in the initial description of a genetic progression model revealed LOH alterations in premalignant lesions advancing toward malignancy [9].

The only possibility of completely preventing cancer is to eliminate (e.g., by apoptosis) all of the initiated and more-advanced premalignant cells and the carcinogenic exposure (e.g., tobacco). Eliminating all premalignant cells without stopping carcinogenic exposure may reset the 20-year carcinogenic clock but will not completely prevent or eliminate cancer risk. The use of retinoids in head and neck carcinogenesis is the best model of the molecular basis and provides the clinical proof-of-principle of cancer delay. Treating premalignancy in its earliest stages could extend the latency period by prolonging the intervals between more of the genetic events along the cancer development pathway [10]

The aim of the current study is to develop molecular assays to improve the early detection of a premalignant lesions.

Materials and methods

The present study included 170 individuals who were admitted to the Ear, Nose and Throat department, Sohag University Hospital in the period between April 2001 and March 2003. The individuals having precancerous lesions of larynx, pharynx, oral cavity, and nose, and SCC of larynx, pharynx, esophagus, and nasopharynx. While the reference objects (control) included 50 individuals (The control patients were cases admitted for ear surgery or simple nasal surgery, from whom we took biopsy from mucosal lining of nasopharynx).

Seventy nine tissue samples of some HNSCC and 41 precancerous tissue samples, were collected from the patients before treatment. Samples were immediately frozen and stored at −80°C. All specimens subjected to the histopathological examination for diagnosis and grading.

Methods

All observed individuals were subjected to the following diagnostic work up. Careful history taking reporting age, sex, special habits as smoking, complaint, and duration of the disease. Assessment of the general condition of the patient; pulse, blood pressure and temperature and any associated lesions or clinical manifestations of distant metastasis and manifestations of nodal involvement were reported. Then, a local ENT examination was performed stressing on nasopharyngeal examination, oropharyngeal examination, indirect laryngoscopy, and lymph node assessment. They were subjected to endoscopy to detect the site and extent of the tumor and to take biopsy for histopathological examination. Full operative data were recorded.

Microsatellites analysis

Selection of chromosomal loci for microsatellite analysis

We investigated LOH by using six microsatellite markers located at chromosomes 3p, 9p, 17p, and 5q. These markers were selected because they frequently demonstrate LOH in HNSCCs and precursor lesions [9, 11, 12]. The following markers were used: D3S1286, D9S171, D9S753, D17S654, D17S695, and CFS1-R. Primer sequences were obtained from the Genome Database for all of these markers (http://www.gdb.org/). These STRs markers were selected for their known propensity for alteration in allelic length (MS1) and LOH. Polymerase Chain Reaction (PCR) products were run on a 6% polyacrylamide gel, developed by sliver staining. Paired normal/lesion samples were tested for LOH (deletion of one allele) and MSI (alteration in the position of one allele by one or more bp). MSI was defined as an alteration

of length of an allele in one or more microsatellites for a particular sample. LOH was defined as a reduction in intensity of an allele to <50% of a retained allele.

DNA extraction and microsatellite analysis

Samples from the premalignant and malignant lesions were obtained from the Hospital. Samples deemed positive for either MSI or LOH were independently reconfirmed by two occasions.

Genomic DNA was isolated from paraffin blocks and represented both precancerous and SCC tissue samples using Promega Wizard Genomic DNA Purification Kit A1120 (Promega, WI, USA) according to the technical manual instructions.

LOH reading and interpretation

Loss of heterozygosity was defined as either complete or near complete loss of a band in the tumor sample relative to the corresponding normal DNA. Convincing evidence of a homozygous deletion in a tumor sample was not observed at any of the six markers used.

Statistical methods

All correlations were performed using a two-tailed Fisher's exact and Qui-square *t*-tests. Linkage analysis of STRs was carried out using *GenePop* Software (Version 3.1, 1997) program.

Results

Regions of chromosomal loss are suspected to encode tumor suppressor genes, the loss of which confers a cellular growth advantage. For each chromosomal locus, individuals will have two alleles, one contributed by each parent. While occasionally parents may provide identical genetic contributions at a given locus (homozygosity), often slight differences are observed among alleles (heterozygosity).

Of the six microsatellites that were successfully amplified, five proved to be polymorphic. The other one microsatellite, CFS1-R, consistently produced spurious banding patterns when the PCR product was electrophoresed on an acrylamide gel. The banding patterns produced by these primers were similar to the shadow banding often seen in microsatellite typing; however, the bands were numerous [4–7] and the intensity of the bands decreased with size, relative to the main band. Consequently, scoring of alleles was not possible using these primer pairs, even though there was apparent allelic variation as judged from main band size heterogeneity in samples from different individuals.

The six primer pairs D3S1286, D9S171, D9S753, D17S654, D17S695, and CFS1-R produced readily scorable allelic variation. Each polymorphic microsatellite was typed in the three sampled groups and the number of alleles present at each locus in each group was determined (Tables 1, 2, 3).

Polymorphism Information Content (PIC) values, allele frequencies, and overall heterozygosities in the three groups are reported for each locus in Tables 1, 2, and 3. PIC values are used to quantify the level of polymorphism for a particular marker.

Table 1 Total number of alleles in each loci, frequencies, LOH, and MSI values of each microsatellite markers in HNSCC patients

Loci	Allele marker total number	PIC value	LOH (%)	MSI value
HNSCCs				
D3S1286	22	0.1206	87.34	38.24
D9S171	9	0.1301	79.74	25.83
D9S753	16	0.2816	67.09	18.46
D17S654	9	0.0713	53.17	13.06
D17S695	37	0.4518	63	5.05
CFS1-R	28	1.0000	100	5.89

Table 2 Total number of alleles in each loci, frequencies, LOH, and MSI values of each microsatellite markers in premalignant patients

Loci	Allele marker total number	PIC value	LOH (%)	MSI value
Premalignant				
D3S1286	12	0.2161	73.17	28.82
D9S171	12	0.2173	46.43	21.91
D9S753	11	0.5914	37	15.10
D17S654	0	0.0000	0.00	0.00
D17S695	13	0.6331	36.59	5.06
CFS1-R	10	1.0000	100	9.31

Table 3 Total number of alleles in each loci, frequencies, LOH, and MSI values of each microsatellite markers in control group

Loci	Allele marker total number	PIC value	LOH (%)	MSI value
Control				
D3S1286	11	0.1341	14.18	8.97
D9S171	7	0.7162	19.41	7.95
D9S753	10	0.1360	17.06	3.13
D17S654	6	0.2371	12.41	20.99
D17S695	13	0.3913	19.24	20.31
CFS1-R	14	0.5212	13.10	14.67

Table 4 LOH frequencies of six polymorphic microsatellite markers at premalignant and HNSCCs

Marker no.	Name	Chromosome location	Premalignant	HNSCCs
1	D3S1286	3pter-3p24.2	30/41 (73%)	69/79 (87%)
2	D9S171	9p21	19/41 (46%)	63/79 (80%)
3	D9S753	9q21.1-22.3	15/41 (37%)	53/79 (67%)
4	D17S654	17p21	0/41 (0%)	42/79 (53%)
5	D17S695	17p13	15/41 (37%)	50/79 (63%)
6	CFS1-R	5q33.3-q34	41/41 (100%)	79/79 (100%)
Total LOH frequencies[b]			120/246 (49%	356/474 (75%)

[b] The total number of LOH frequencies in study groups

By summing the frequencies multiplied by the probability that all three groups will be informative. Highly informative loci have PIC values greater than 0.5, reasonably informative loci between 0.25 and 0.5, and slightly informative loci have PIC values of less than 0.25. These values are used to evaluate the utility of markers used in genome mapping studies.

There was a trend toward a higher prevalence, of these alterations in the more histopathologically severe lesions, from 49% in the premalignant lesions to 75% of the malignant cancer samples (Table 4) and 31% in control samples.

For informative markers, the most frequent microsatellite markers with LOH were detected at loci *CFS1-R*, *D3S1286, and D9S171*, respectively. Additionally, in informative biopsies from both types of patients, the least frequent microsatellite marker for LOH detection was *D17S654*, in premalignant, malignant, and control samples.

One-microsatellite marker, D17S654 failed to amplify premalignant DNA. PCR conditions (e.g., MgCl, annealing temperature, and primer concentration) were varied in attempts to induce amplification, but the primers would not amplify a fragment of correct size. Stringent conditions produced no bands when PCR product was electrophoresed on a sequencing gel, while more lab conditions resulted in many nonspecific bands.

There is a statistically significant difference between premalignant and HNSCCs in the frequency of LOH among the informative cases for all of the six markers combined (x^2; $P < 0.001$).

All markers exhibited LOH and MSI at a moderate to high frequency. LOH were found in more than 60% of the tumors for four markers (D3S1286, D9S171, D9S753, and D17S695). Among the markers analyzed on the long arm, CFS1-R at 5q33.3-q34 displayed the highest frequency of LOH (100%). Interestingly, markers D9S171, D9S753, and D17S695 exhibited increase in LOH from 46, 37, and 37% in precancerous lesions to 80, 67, and 63% in malignant lesions respectively. Thus, it appears that the region 9p21.1-22.3 and 17p13 are prone to genetic instability in HNSCC tumors. In contrast, the absence of LOH in D17S654 in premalignant and its occurrence in malignant suggests that it is a late event and associated with tumor progression.

Discussion

The development of molecular markers is needed to improve diagnosis and prognosis of disease and to assess tumor progression in HNSCC patients.

The overall results of the present study suggest that the presence of large genetically altered events (i.e., chromosomal aberrations, STRs mutations) is a risk factor for the development of HNSCCs. Therefore, the analysis of genetically altered mucosa might identify the subgroup of patients at high-risk for developing HNSCC. Other parameters should be taken into account as well, such as follow-up time [13] and certain specific genetic alterations. If such a high-risk patient group can be identified, chemoprevention or gene therapy is potential approaches to prevent the development of HNSCCs.

Loss of heterozygosity and MSI have been implicated as a possible means of early detection of head and neck malignancy [7]. A genetic progression model has also formed the basis of demonstrating a characteristic pattern of LOH in premalignant lesions of the head and neck [9]. Furthermore, early microsatellitic loss at chromosomes 3p and 9p is related to progression to head and neck malignancy. However, MSI in premalignant lesions of the head and neck has not been studied independently to identity a temporal pattern of progression. It is important to understand the timing of disease alterations in HNSCC because their incorporation into detection strategies through molecular methods offers the possibility of better treatment modalities aimed at cure rather than salvage.

Microsatellite markers are small repeating DNA sequences found in the introns (noncoding regions) of a gene. PCR amplification of these repeat sequences provides a rapid method for assessment of LOH and facilitates mapping of tumor suppressor genes [6].

Screening for cancer cells in a clinical sample may be possible when specific mutation in a target gene is not known by targeting tumor-specific alterations in microsatellite

repeat sequence [13]. The location of a number of STRs markers has been described the primers are available for their PCR amplification. If a clinical sample contains an altered number of repeats in comparison to normal tissue, a band will appear in a unique position on the gel. The presence of such a tumor-specific band in DNA isolated from a clinical sample such as peripheral blood, or saliva indicates the presence of clonal cancer cells. The intensity of this band in the test sample is proportional to the relative number of cancer cells present. As few as one tumor cell in the background of 200 normal cells can be detected using this approach.

In the present study, an allelic imbalance was examined mainly in six regions (3pter-3p24.2, 5q33.3-q34, 9p21, 9q21.1-22.3, 17p13, and 17p21) of chromosomes 3, 5, 9, and 17 in premalignant, and HNSCC patients compared with healthy controls.

The analysis showed that markers CFS1-R at 5q33.3-q34, D3S1286 at 3pter-3p24.2, and D9S171 at 9p21 exhibited the highest frequency of LOH (100, 87.34, and 79.74%, respectively). It was necessary to test multiple markers at several loci because of the frequent expression of MSI that confounded our mapping efforts in HNSCC with replication errors.

Partridge et al. reported that LOH at 3p is one of the most common events in HNSCC and has been associated with poor prognosis [14].

Van Rees et al. reported LOH at 3p, 9p, and 17p were identified as early events occurring in benign and preinvasive lesions of the aerodigestive tract [15]. In another study, LOH was observed at 9p21 in the early event, at 17p13.1, 3p25, and 3p14.2 in the intermediate event and 8p21.3-p22 in the late event of laryngeal carcinogenesis [16]. Li et al. reported significant LOH occurred at 3p21 (32%), 3p25-26 (56%), 8pter-21-1 (31%), 13q14 (27%), and 17p12 (45%) [17]. LOH at more than two loci was associated with poor prognosis [17].

It has been suggested that allelic imbalance (generated by LOH) on the short arms of chromosomes 3, 9, and 17 indicates the location of tumor suppressor genes associated with the early stages of other cancers such as in lung cancer development, there is LOH at loci on the short arm of chromosome 3 [18]. This pattern of microsatellite alterations and LOH may be specific for different types of cancer. The high incidence of these changes on chromosomes 3, 5, 8, 9, 10, 11, 17, and 20 have been described in lung cancer specimens. Nawroz et al. defined 29 primary head and neck tumors for allelic loss. They found that chromosomes 3, 11q, 13q, and 17p exhibited loss in over 50% of all informative cases, while chromosomes 4, 6p, 8, 14q, and 19q displayed loss in greater than 35% of all cases tested [19]. In the same study, the high frequency of allelic loss was found on chromosome 9p where 21 of 29 (72%) tumors had LOH for at least one polymorphic marker on this arm.

Loss of heterozygosity was reported at 9p21-p22 in 72% of tumors [20]. Recently, Partridge et al. identified five areas in the region of allelic imbalance at chromosome 3p that might harbor tumor suppressor genes, along with two areas at 8p and 9p, respectively [11]. These authors also identified significantly greater allelic imbalance in patients with TNM stage 4 disease compared with stages 1–3.

The present results indicated that, it is possible that a similar mechanistic defect occurs in the progression of precancerous to malignancy, which is independent of LOH.

In 27 of the 41 precancerous lesions analyzed, only one genomic alteration was detected, whereas five or more genetic changes were found in the other 14 lesions. The most frequent losses identified included those on 3p, 9p, 5q, and 17p which had previously been reported in precancerous lesions by LOH analysis [9, 13, 20].

Certain genetic events (3p21, 9p21, and 17p13 LOH) tend to occur earlier on the progression pathway, but others, usually late-occurring event like in 17p21, in the time course of progression. This finding suggested that 9p LOH occurs very early in HNSCC tumor progression. Besides 9p21, 3p21 was found to have LOH in many early lesions, the frequency of loss of these loci plateaued in carcinoma in situ, and was no more common in invasive disease. The study of Califano et al. demonstrated that losses at 8p and 4q were found only in a few in situ lesions and were significantly more common in invasive cancer, making them likely late events [9]. Based on these studies, LOH of 17p, 11q, 13q, 14q, 6p, and 8q were judged to be intermediate events. It is not possible to know the exact order of genetic events in any individual cancer. Indeed, the order of events probably varies from one individual to another, as has been demonstrated in colon cancer [21].

It also may differ in different parts of the world due to the different factors for carcinogenesis which reflect the ethnic origin.

Conclusion

Examination of LOH could improve diagnosis, adds additional confidence, in HNSCC by DNA extraction from suspicious lesions in high-risk groups (smokers and alcoholics) and LOH at 3p/9p seems to be of particular value for early detection and definition of progression risk. If there are high percent of LOH at these chromosomes, active intervention should be done (chemoprevention and regular follow up head and neck examination for very early detection and management).

References

1. Parkin DM, Loara E, Muir CS (1988) Estimates of the world wide frequency of sixteen major cancers in 1980. Int J Cancer 41:184–197
2. Hunter KD, Parkindon EK, Harrison PR (2005) Profiling early head and neck cancer. Nat Rev Cancer 5:127–135
3. Nagai MA (1999) Genetic alterations in head and neck squamous cell carcinomas. Braz J Med Biol Res 32(7):897–904
4. Hibi K, Robinson CR, Booker S, et al (1998) Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res 58:1405–1407
5. Goessl C, Heicappell R, Munker R, et al (1998) Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. Cancer Res 58:4728–4732
6. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D (1996) Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 2:1035–1037
7. Spafford MF, Koch WM, Reed AL, et al (2001) Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis. Clin Cancer Res 7:607–612
8. Field JK, Kiaris H, Risk JM, et al (1995) Allotype of squamous cell carcinoma of the head and neck: fractional allelic loss correlates with survival. Br J Cancer 72:1180–1188
9. Califano J, van der Riet P, Westra W, et al (1996) Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 56:2488–2492
10. Lippman SM, Hong WK (2002) Cancer prevention by delay Commentary re: J. A. O'Shaughnessy et al Treatment and prevention of intraepithelial neoplasia an important target for accelerated new agent development. Clin Cancer Res 8:305–313, 314–346
11. Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J (1999) Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. Int J Cancer 83:318–325
12. Gupta VK, Schmidt AP, Pashia ME, Sunwoo JB, Scholnick SB (1999) Multiple regions of deletion on chromosome arm 13q in head-and-neck squamous-cell carcinoma. Int J Cancer 84:453–457
13. Mao L, Lee J, Fan YH, et al (1996) Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nat Med 2:682–685
14. Partridge M, Emilion G, Langdon JD (1996) Loss of heterozygosity at 3p correlates with a poor survival in oral squamous cell carcinoma. Br J Cancer 73:366–371
15. van Rees BP, Cleton-Jansen A, Cense HA, et al (2000) Molecular evidence of field cancerization in a patient with 7 tumours of the aerodigestive tract. Hum Pathol 31(2):269–271
16. Yoo WJ, Cho SH, Lee YS, et al (2004) Loss of heterozygosity on chromosomes 3p,8p,9p and 17p in the progression of squamous cell carcinoma of the larynx. J Korean Med Sci 19(3):345–351
17. Li X, Lee NK, Ye Y, et al (1994) Allelic loss at chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer. J Natl Cancer Inst 86:1524–1529
18. Sundaresan V, Ganly P, Hasleton P, et al (1992) p53 and chromosome 3 abnormalities, characteristic of malignant lung tumours, are detectable in preinvasive lesions of the bronchus. Oncogene 7:1989–1997
19. Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D (1994) Allelotype of head and neck squamous cell carcinoma. Cancer Res 54(5):1152–1155
20. El-Naggar AD, Hurr K, Batsakis JG, Luna MA, Goepfert H, Huff V (1995) Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous carcinoma. Cancer Res 55:2656–2659
21. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61:759–767