Analysis of Microsatellite Instability in Laryngeal Squamous Cell Carcinoma

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Abstract

Introduction and objectives: The literature on the involvement of microsatellite instability in head and neck squamous cell carcinoma shows great variability, probably due to differences in the testing methods. Using a consensus detection system, we aimed to reach a reliable estimate of microsatellite instability prevalence in a subset of head and neck squamous cell carcinoma cases.

Methods: The microsatellite instability status of 43 patients with previously untreated primary laryngeal squamous cell carcinomas was analysed by a multiplex polymerase chain reaction assay including 5 mononucleotide repeat markers.

Results: Thirty-six cases showed a stable phenotype or a microsatellite stable phenotype (83.7%) and 7 cases (16.3%) showed a microsatellite instability-positive phenotype. One case showed instability in 3 of 5 markers, 1 case in 2 markers and 5 cases in 1 marker. The microsatellite instability-positive and stable cases did not differ with respect to age, tumour stage, lymph node or distant metastases.

Conclusions: Our data showed that a proportion of laryngeal squamous cell carcinomas are microsatellite instability positive. Knowledge of microsatellite instability patient status will allow adjusting anticancer therapy at an individual level.

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Análisis de la inestabilidad de microsatélites en el carcinoma escamoso de laringe

Resumen

Introducción y objetivos: La literatura sobre la participación de la inestabilidad de microsatélites en el carcinoma de células escamosas de cabeza y cuello muestra una gran variabilidad, probablemente debido a las diferencias en la metodología de las pruebas. Utilizando
un sistema de detección consensuado, nos planteamos como objetivo llegar a una estimación fiable de la prevalencia de la inestabilidad de microsatélites en un subconjunto de carcinomas de células escamosas de cabeza y cuello.

**Métodos:** Se analizó el estado de inestabilidad de microsatélites en 43 pacientes no tratados previamente y diagnosticados de un carcinoma primario de células escamosas de laringe mediante una prueba de PCR múltiple, incluyendo 5 marcadores repetidos de mononucleótidos.

**Resultados:** En 36 casos se observó un fenotipo estable o microsatélites estables (83,7%), y en 7 casos (16,3%) se mostró un fenotipo positivo de inestabilidad de microsatélites. Uno de los casos mostró inestabilidad en 3 de los 5 marcadores, otro mostró inestabilidad en 2 marcadores y 5 casos en un marcador. Entre los casos de inestabilidad de microsatélites positiva y los casos estables no hubo diferencias con respecto a la edad, el estadio del tumor, la afectación de los ganglios linfáticos o las metástasis a distancia.

**Conclusiones:** Nuestros datos muestran que una parte de los carcinomas de células escamosas de laringe presentan inestabilidad de microsatélites. El conocimiento sobre el estado de inestabilidad de microsatélites de los pacientes permitirá el ajuste de la terapia anti-cancerígena a nivel individual.

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**Introduction**

Cancer develops as a multistep process in which genetic changes accumulate. One of these steps can cause damage to the genes involved in maintaining the integrity of DNA. When such damage occurs to mismatch repair mechanisms it originates mutations or instability within repetitive DNA sequences known as microsatellite instability. Microsatellite instability is characterised by deletions or expansions in small repetitive sequences (1–5 nucleotides repeated between 5 and 100 times). The majority of these sequences appear in noncoding DNA, but they can also originate in important genes such as TGF-βRII, BAX and IGF2R. Microsatellite instability was first described in hereditary nonpolyposis colorectal cancer and in sporadic colon cancer, and is present in approximately 15% of cases.

Regarding squamous cell carcinoma of the head and neck (SCCHN), microsatellite instability has been found in the literature in varying frequencies, ranging between 1% and even 100%. The reason for these inconsistent results is probably due to a difference in the methodologies employed and possibly also to different sub-locations of squamous cell carcinoma of the head and neck (tongue, larynx, oral cavity, nasal passages) being grouped together. Knowledge of microsatellite instability status in tumours is clinically important because it affects the processing of DNA damage induced by the chemotherapeutic drugs employed to combat squamous cell carcinoma of the head and neck, such as cisplatin, carboplatin and 5-fluorouracil.

Microsatellite instability is important in certain tumours, and laryngeal squamous cell carcinomas could be an example. Microsatellite instability is probably important in pre-malignant laryngeal lesions, having been found with high frequency among young patients with squamous cell carcinoma of the head and neck. Therefore, it is important to know the real prevalence and consequences of positive microsatellite instability in laryngeal squamous cell carcinoma.

This work aims to explore the prevalence of microsatellite instability by using a detection kit with verified reliability for laryngeal squamous cell carcinoma, relate the findings with clinical and pathological parameters and carry out a follow-up of the information.

**Methods**

**Tumoural Samples**

We obtained samples of laryngeal squamous cell carcinomas from 43 patients through specimens of surgical resections avoiding necrotic areas, which were stored in liquid nitrogen. We also obtained informed consent from all patients, after the study was approved by the ethics committee of our institute. All patients underwent radical surgery, and in all cases the resection margins were free of tumours. A total of 4 patients had received prior radiotherapy. The mean age was 60 years (range 43–80 years). Within the sample, 10 tumours were at stage I, 13 at stage II, 13 at stage III and 7 at stage IV. In addition, 16 tumours were well differentiated, 14 moderately differentiated and 11 poorly differentiated. Follow-up information was available throughout a mean period of 46 months (range 0–100 months). Regarding recurrences, 1 patient developed a secondary primary tumour, 11 suffered locoregional recurrence and 2 suffered distant metastases. The complete list of clinical data is presented in **Table 1**.

**Analysis of Microsatellite Instability**

The tumoural DNA was extracted using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany). We expanded approximately 2 Ng of tumoural DNA following the recommendations of the manufacturer. We performed a multiplex PCR using a microsatellite instability test kit (Promega Biotech Ibérica, Barcelona, Spain), consisting of 2 primers for 5 nearly nonmonomorphic mononucleotide markers: BAT-25, BAT-26, NR-21, NR-24 and MONO-27 (Table 2). The PCR mixture contained 17 µl of nuclease-free water; 2.5 µl of Goldst.r 10× buffer; 2.5 µl of 10× multiplex primer mix; 0.5 µl of AmpliTag Gold DNA polymerase at 5 units/µl and 2.5 µl of DNA at 0.8 Ng/µl. We
Results of the analysis of 43 LSCC carcinomas are summarised in Table 1. The characterisation of LSCC carcinomas for microsatellite instability (MSI) was performed using Genescan software (Applied Biosystems, Warrington, UK). Changes in the size of alleles with 3 or more base pairs or one or more of the 5 markers were scored as positive for microsatellite instability. Conversely, changes in the size of alleles with 2 or less base pairs were scored as microsatellite stability. A negative control sample of control DNA was provided to the Promega kit. We performed a positive control experiment using DNA from
the colorectal cancer patient with positive microsatellite instability that had reflected changes in the 5 mononucleotide markers.

Statistical Analysis

The possible correlations between the status of microsatellite instability and clinical parameters were statistically analysed using the software package SPSS 12.0 for Windows (SPSS Inc., IL, USA), as well as the Student’s t-test and the Fisher $\chi^2$ exact test. We performed a Kaplan–Meier analysis to estimate survival, comparing the survival distributions through the log-rank test. Statistical significance was considered for values of $P<.05$.

Results

All the tumours provided some interpretable PCR products. Their sizes were within the range suggested by the manufacturer. In 36 cases, microsatellite stability analysis revealed a stable phenotype (83.7%), whereas in 7 cases (16.3%) it revealed a positive phenotype of microsatellite instability. LSCC1 showed microsatellite instability in 3 of the 5 markers for loci BAT-25, BAT-26 and MONO27, LSCC21 in BAT-25 and MONO27, LSCC2 in BAT-26, LSCC11 and LSCC27 in BAT-25 and LSCC42 and LSCC14 in NR-21 (Table 1). Two examples are shown in Fig. 1.

We used the Student’s t-test and the $\chi^2$ and Kaplan–Meier statistics to contrast the cases of microsatellite stability and instability. We found no difference with respect to age of patients at diagnosis, tumour stage, lymph nodes or distant metastases (Table 3). We observed a trend in cases of microsatellite instability, which showed a weaker histopathological differentiation than cases of microsatellite stability, with figures of 57% and 25%, respectively, although this finding was not significant.

Discussion

Since its discovery in the early 1990s, various techniques have been used to monitor microsatellite instability. In an attempt to standardise the method, an NCI (National Cancer Institute) workshop in 1997 recommended a panel of 5 microsatellite markers, known as the Bethesda panel. However, this panel created problems, especially due to the inclusion of repeated dinucleotide markers. Such limitations were discussed during an NCI workshop in 2002, which led to a review of the recommendations for microsatellite instability tests. The Promega kit used in this study was based on the consensus recommendations and only contained repeated mononucleotide markers. Bacher et al. showed that this panel is more sensitive and more specific than the Bethesda panel. This methodology was used by Yalniz et al. to develop a consensus on microsatellite instability markers in squamous cell carcinoma of the head and neck and we obtained a similar result for laryngeal carcinomas. Most of the literature on microsatellite instability in squamous cell carcinoma of the head and neck is based on methodologies including dinucleotide and even trinucleotide markers, which often tend to produce false positive results. For this reason, it is very difficult to compare our data with those from previous studies. The percentage of positive cases obtained in our series (16.3%) may suggest that this mechanism plays a role in a subset of tumours. We found no significant differences between cases of microsatellite stability and instability in relation to tumour stage, lymph nodes, metastasis or clinical outcome. This might suggest that microsatellite instability takes place at a very early stage in tumour development. This agrees with microsatellite instability findings in premalignant laryngeal lesions and with its very high frequency among young patients with squamous cell carcinoma of the head and neck. In our study, cases of microsatellite instability presented a frequency of poor histopathological differentiation relatively above that of tumours with microsatellite stability, with percentages of 57% and 25%, respectively. These results are similar to those reported by Nash et al. for colon cancer, in which microsatellite instability was associated with poorly differentiated cancers.

Although this has not been clearly established, environmental factors such as oxygen radicals, smoking or diet may also play a role in the development of microsatellite instability. Microsatellite instability has been associated with methylation of CPG islands and smoking in sporadic colon cancer, bronchial epithelium and lung cancer. It has been reported that mismatch repair gene MLH1 is frequently silenced by promoter methylation. There is speculation that this association also exists in laryngeal squamous cell cancer, where the majority of patients are smokers, defining a specific subgroup of patients.

The correct functioning of DNA repair pathways is important in the choice of anti-cancer therapy. Mismatch repair is included in the processing of DNA damage caused by the use of various types of chemotherapy drugs, such as monofunctional alkylating agents, bifunctional alkylating agents (cisplatin and carboplatin), antimetabolites and fluoropyrimidine (5-fluorouracil and fluorodeoxyuridine), commonly used in the treatment of squamous cell carcinoma of the head and neck. Other options include surgery, such as micro-CO$_2$ laser surgery for organ preservation in the treatment of advanced laryngeal carcinomas. During in vitro experiments, cells with stable microsatellites proved 2 to 100 times more sensitive to these drugs than cells with unstable microsatellites. In fact, animal studies on different tumour types suggest that cells with microsatellite instability have a weaker response to cisplatin, carboplatin and methylating agents. By contrast, new drugs are being developed that utilise the microsatellite instability
Figure 1  Example of control without microsatellite instability and positive cases. Arrows indicate extra peaks, marking changes at specific loci of repeated DNA sequences.

Table 3  Differences Between Microsatellite Stability and Instability With the Same Clinical Parameters.

<table>
<thead>
<tr>
<th></th>
<th>Mean Age</th>
<th>Recurrences</th>
<th>Lymph Node Involvement</th>
<th>Metastasis</th>
<th>Deceased Due to Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSI (n=7)</strong></td>
<td>56.3</td>
<td>28.57% (2 of 7)</td>
<td>14.29% (1 of 7)</td>
<td>14.29% (1 of 7)</td>
<td>28.57% (2 of 7)</td>
</tr>
<tr>
<td><strong>MSS (n=36)</strong></td>
<td>61.2</td>
<td>36.11% (13 of 36)</td>
<td>30.56% (11 of 36)</td>
<td>2.78% (1 of 36)</td>
<td>27.78% (10 of 36)</td>
</tr>
</tbody>
</table>

MSI: microsatellite instability; MSS: microsatellite stability.

Conclusion

We conclude that some laryngeal squamous cell carcinomas have positive microsatellite stability. Knowledge of microsatellite instability status of patients will enable a better adjustment of anti-cancer therapy at an individual level. Microsatellite instability tests may be clinically and economically useful in laryngeal squamous cell carcinoma.

Financing

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Conflict of Interests

The authors have no conflicts of interest to declare.

References