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Evaluation of tumor microsatellite instability in laryngeal cancer using five quasimonomorphic mononucleotide repeats and pentaplex PCR

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ABSTRACT

Purpose: To determine microsatellite instability (MSI) in squamous cell carcinoma of the larynx.
Material and Methods: The study was performed in a group of 30 male patients, aged 42–47 years. All patients underwent total laryngectomy with lymph nodectomy. Histologically all tumors were squamous cell carcinomas. Detection of MSI was based on comparison of allelic profiles generated from amplification of matching normal and tumor DNA.
Results: The appearance of novel alleles in the tumor DNA indicated microsatellite instability. MSI analysis showed a microsatellite stable phenotype in 23 cases (77%).
Conclusions: MSI may not contribute to the development of squamous cell carcinoma of the larynx.

Key words: squamous cell carcinoma of the head and neck (SCCHN), microsatellite instability (MSI), squamous cell carcinoma of the larynx (SCCL)

INTRODUCTION

Human cancers develop by accumulating a range of somatic genetic changes throughout their progression [1], however the molecular basis for these changes remains unclear in the majority of cancers. It has been postulated that genetic damage affecting the majority of autosomal chromosome arms, including activation of oncogenes and/or inactivation of tumor suppressor genes (TSGs), possibly acting in conjunction with an impaired capacity of DNA repair mechanisms, may be a factor in the development of squamous cell carcinoma of the head and neck (SCCHN) [2].

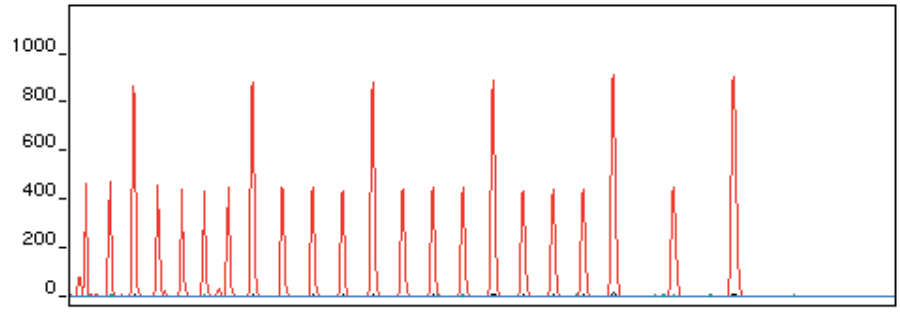
Defective human DNA mismatch repair mechanism manifests itself as the phenomenon of "microsatellite instability" (MSI) and is deemed a separate entity to allelic imbalance or loss of heterozygosity (LOH). MSI is characterized by small deletions or expansions within short tandem repeats in tumor DNA as compared with matching normal DNA. Tumors with MSI have been classified as "microsatellite unstable" or "replication error positive" (RER+) [3,4].

MSI has been shown to be one of the important features of cancer cells, due to replication errors (RER) leading to accumulation of mutations in cancer-associated genes. The phenomenon of MSI in human carcinomas resulting from the inactivation of MMR genes was first shown in hereditary non-polyposis colon cancer (HNPCC) [5]. More than 80% of HNPCC and about 30% of sporadic colon cancers demonstrate a high frequency of MSI. [5-9].

Although several hundred different microsatellite markers have been used for MSI analysis, it is unclear how many markers and which chromosomal loci should be used to evaluate MSI, and what percentage of unstable microsatellite define a tumor as MSI [2].

It has been suggested that, like TSGs, inactivation of human DNA mismatch repair genes requires two steps [10]. The first event is presumed to be a deleterious mutation, and the second mutational event was initially reported as an allelic imbalance at the hMLH1 locus in HNPCC patients [11], and

Figure 1. **Internal Lane Standard 600 (ILS 600).**



later in sporadic colorectal cancer (CRC) [12], sporadic breast cancer [13], non-small cell lung cancer (NSCLC) [14] and cervical squamous cell carcinoma [15]. It has been considered that, in microsatellite instability positive (MSI+) tumors, LOH might occur frequently at the DNA mismatch repair (MMR) gene loci, thereby inactivating one MMR allele in a way similar to allele loss at tumor suppressor gene loci.

Seven human mismatch repair genes have been located, all homologous to known MMR genes found in the bacteria *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. The human homologue of the bacterial MutS gene, hMSH2, was the first MMR gene to be mapped and isolated on chromosome 2p16 [16,17]. Three human homologues of the *MutL* gene; hMLH1, hPMS1 and hPMS2 have loci at chromosome 3p21, 2q31-33 and 7p22, respectively [18, 19, 20]. Since then two new MutS homologues have been reported, hMSH3 and hMSH6, located at 5q11-13 and, within 1 mega base of hMSH2, at 2p16, respectively [21-24] and a new MutL homologue, hMLH3, is located at 14q24.3 [25]. Although a number of reports exist on MI in various tumors, its real significance in tumor progression is unknown.

Among human malignancies, head and neck cancer is the sixth most common cancer type in the world [26]. Squamous cell carcinoma of the larynx (SCCL) is the most common malignancy of the group of head and neck squamous cell cancers and accounts for approximately 5% of all cancers. Epidemiological studies suggest that the incidence of and mortality from SCCL is increasing, particularly in developed countries [27,28]. In the etiology of larynx cancer both environmental and multiple genetic alterations are involved [29,30]. Although abundant cytogenic and molecular genetic data on larynx cancer have been accumulated, the genetic mechanisms involved in the pathogenesis and progression of the disease remain unknown [31]. It has also been hypothesized that larynx cancer development is related to widespread genomic instability [32].

MATERIAL AND METHODS

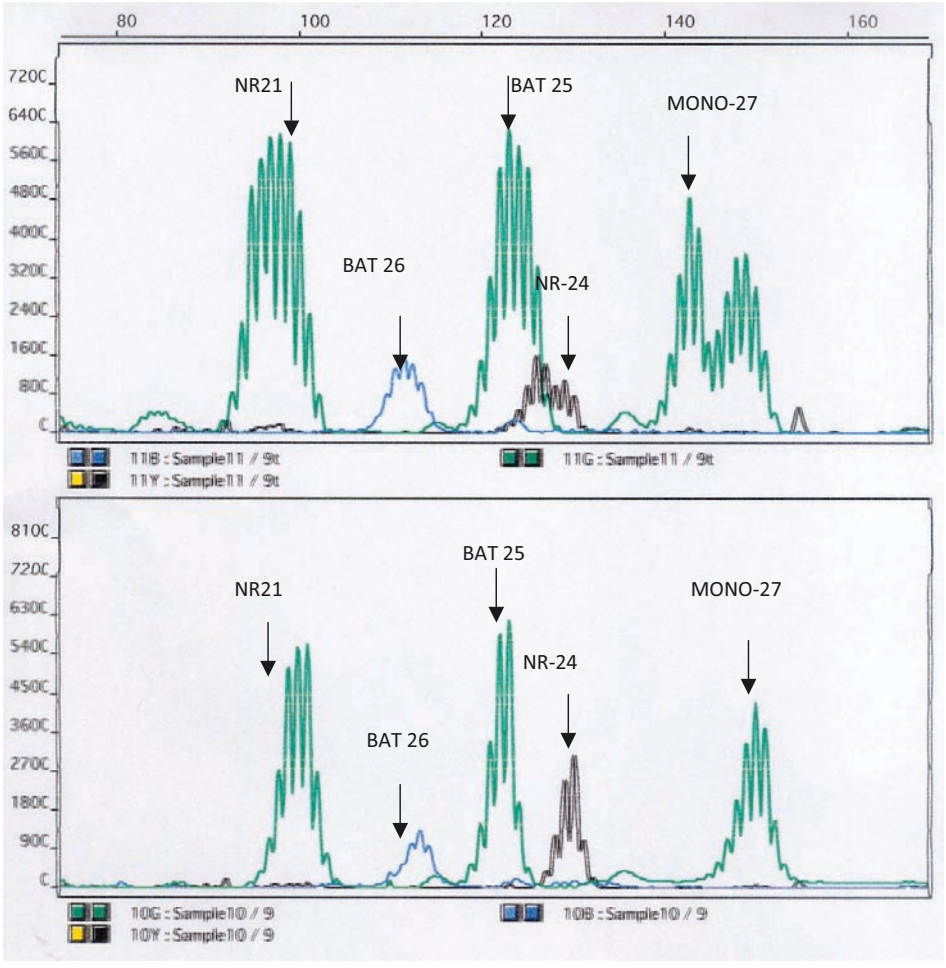
The study was performed in a group of 30 male patients, aged 42–77 years. All patients underwent total laryngectomy with lymphonodectomy. Tumor tissue specimens and reference peripheral blood samples were obtained during surgical resections. Histologically all tumors were squamous cell carcinomas. On histopathologic specimens, G1 grade cancers were diagnosed in 13 patients (43%), G2 grade cancers in 9 patients (30%) and G3 grade cancers in 8 patients (27%). Lymph node metastases were found in 15/30 cases, with G1 in 5 patients, G2 in 5 patients and G3 in 5 patients.

Tumor tissue specimens and reference peripheral blood samples were obtained during surgical resections. The standard organic method was used for DNA isolation. Additional microcolumn purification was performed when necessary. Genotyping was performed in a 310 ABI Prism Genetic Analyzer (Applied Biosystems, USA) using the GeneScan Analysis v3.7 and Genotyper v3.7 software. Five fluorescently-labeled mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24 and MONO-27 and two pentanucleotide repeat markers PentaC and PentaD were co-amplified using the MSI Analysis System (Promega). The mononucleotide repeat markers were selected for high sensitivity and specificity to alterations in tumor samples with mismatch repair defects. Mononucleotide makers included in the MSI Analysis System are quasimonomorphic, which simplifies data interpretation. All patients in the study were found to be homozygous for the same common allele for a given marker.

Two pentanucleotide markers PentaC and PentaD were added to identify sample mix-ups and/or contamination [33]. Detection of MSI was based on comparison of allelic profiles generated from amplification of matching normal and tumor DNA. The appearance of novel alleles in the tumor DNA indicated microsatellite instability. ANOVA was performed for statistical analysis. Tumors with instability in two or more of these markers were defined as MSI-High (MSI-H), whereas those with one unstable marker were designated as MSI-Low (MSI-L). Tumors with no detectable alterations are stable (MSS).

The Internal Lane Standard 600 (ILS 600) contains 22 DNA fragments of 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases in length (*Fig. 1*). Each fragment is labeled with carboxy-X-rhodamine (CXR) and is detected separately (as a fourth color) in the presence of MSI Analysis System-amplified material using the ABI PRISM 310 Genetic Analyzer. The ILS 600 is designed for use in each capillary electrophoresis (CE)

Figure 2. **MSI-positive laryngeal cancer samples (upper panel) matching normal samples (lower panel). The presence of new alleles at loci in the tumor sample (see arrows) that were not present in the normal sample indicates MSI.**



injection to increase precision in analysis when using the MSI Analysis System.

A reference standardized panel of five markers was suggested for MSI analysis by a National Cancer Institute (NCI) workshop in 1997. However limitations resulting from the inclusion of dinucleotide markers, which are less sensitive and specific for detection of tumors with MMR deficiencies compared with other types of markers currently available, have been revealed [5].

RESULTS

MSI analysis showed a microsatellite stable phenotype in 23 cases (77%). Using BAT-26 a low-frequency MSI (MSI-L) type in 5 cases (17%) was found, whereas high-frequency MSI (MSI-H) tumors were found in 2 cases (6%) when using NR-24 and MONO-27 markers. No MSI was revealed at the BAT-25 or NR-21 markers. No significant difference was found between MSI-H and MSI-L/MSS tumors with regard to stage, differentiation or other clinico-pathological parameters. All electropherograms of mononucleotide markers showed a number of less intense stutter peaks at 1bp intervals from the most prominent or true allele peak, generated by polymerase slippage during PCR amplification of short tandem repeats. Small pull-up peaks were visible at the baseline, thus thresholds in the GeneScan analysis parameters were preset to compensate for these artifacts. A shift in allele size of 3bp or more in the tumor samples compared with matching normal samples was scored as microsatellite instability (*Fig. 2*).

DISCUSSION

Our study was limited due to a small number of patients. Recent studies indicate that a variety of sporadic tumors exhibit frequent microsatellite instability associated with defects in the DNA mismatch repair pathways. MSI was observed in 23% of our patients. This rate is somewhat lower then previously reported for head and neck cancer, where Demokan et al. observed microsatellite instability in 41% of the patients [26], Wang et al. observed 100% of tumors that had MSI at one site at least and 88% that had MSI at 2 or more loci [32]; and higher than observed for larynx – Sasiadek et al. did not observe MSI

in their paper [34], and Smigiel only at 4.8% [35]. In our study MSI at the BAT-26 marker was the most common observation. The instability at this locus was also observed by other authors [26,35,36]. No instability at BAT-25 was found, which was in contrast to the study of Demokan et al [26]. Data from Hoang et al. confirm the observation that BAT26 is perhaps the best marker for MSI assessment in colorectal cancers [37]. There are no data on the informativeness of the other dinucleotide repeats e.g. NR-21, NR-24 and MONO-27 when evaluating the MSI in laryngeal cancer, although the markers are reported to allow accurate evaluation of tumor MSI status of the hereditary nonpolyposis colorectal cancer syndrome as well as sporadic colon and gastric tumors [33] A reference standardized panel of five markers was suggested for MSI analysis by a National Cancer Institute (NCI) workshop in 1997, however limitations resulting from the inclusion of dinucleotide markers, which are less sensitive and specific for detection of tumors with MMR deficiencies compared with other types of markers currently available, have been revealed [5]. The five quasimonomorphic mononucleotide repeats may be more sensitive for MSI-H tumors than other microsatellite markers and may obviate the need for normal tissue for comparison; this approach requires three or more mutant alleles to indicate MSI-H [38]. It is now widely accepted that MSI assessment should be added as a distinct criterion to refine the diagnosis of HNPCC, which is not standard in head and neck cancers. Finally, the status of the mismatch repair system may be important in predicting tumor response to clinical therapy, because it appears that mismatch repair-deficient cells are resistant to the chemotherapeutic agents cisplatinum, 5-fluorouracil and mephalan, while being more sensitive to gamma-radiation [2]. With this in mind, it is extremely important to reliably assess the MSI status of colorectal tumors in a diagnostic clinical setting. From a study of et Smigiel al [34] and Sasiadek et al [35] it can be concluded that MSI may not contribute to the development of SCCL. Further studies with a larger number of patients are needed to elucidate the contribution of genetic factors, especially MSI, to the development of laryngeal cancer as well as whether quasimonomorphic mononucleotide repeats are useful in detecting MSI of laryngeal cancer.

CONCLUSIONS

MSI may not contribute to the development of SCCL. Further studies with a larger number of patients are needed to elucidate the contribution of genetic factors, especially MSI, to the development of laryngeal cancer as well as whether quasimonomorphic mononucleotide repeats are useful in detecting MSI of laryngeal cancer.

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