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Assessment of microsatellite instability in head and neck cancer using consensus markers

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Abstract Head and neck cancer is the sixth most common cancer in the world and one of the most lethal cancers. Microsatellite instability is an important characteristic of tumor cells and is observed both in presence and absence of mismatch repair gene mutations. The importance of microsatellite instability in head and neck cancer is not well established due to the lack of a consensus panel and selection of different markers, criteria and methodological variances. The main objective of this study was to investigate the performance of a consensus panel of microsatellite repeats by automated fragment analysis. Matched tumor and normal tissue samples from 99 patients were analyzed using five mononucleotide markers. Following PCR the amplified fragments were analyzed by capillary electrophoresis on an ABI 310 genetic analyzer. Microsatellite instability was observed in 26 patients. In 17 patients instability was detected at multiple loci. NR21 and BAT25 were the most frequently altered targets. These two mononucleotide markers could detect all samples displaying high-instability. In this study we describe a standardized fluorescent multiplex PCR combined with computerized analysis, which allows rapid and accurate analysis of a high number of samples and obviates the need to compare tumors with matching normal tissue.

Keywords Microsatellite instability · Head and neck cancer · Mononucleotide markers

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Introduction

Head and neck cancer is the sixth most common type of cancer in the world [1] and is one of the most lethal cancers [2]. Both environmental and genetic factors play an important role in the etiology but the causal relationship between these and tumor development is not yet clear [3]. Therefore, there is considerable effort to identify informative new molecular markers that might facilitate early detection, improve stratification of the patients and help to predict prognosis.

Polymorphic microsatellites are useful genetic markers and important tools for studying genetic changes in tumors. Tandem microsatellite sequences are prone to misalignment and slippage during DNA replication and result in the deletion or insertion of repeating units producing novel alleles of different sizes. Cells with impaired MMR genes are not able to correctly repair replication errors occurring at repetitive structures. Recent studies also indicate that a substantial fraction of sporadic tumors display microsatellite instability (MSI) although most tumors do not harbor mutations in the repair genes [4]. There are hundreds of thousands of microsatellite loci that could potentially be used for MSI analysis [5]. In a previous study we have shown that microsatellite instability and mutations in the hMLH1 and hMSH2 genes may play a significant role in head and neck cancer [6].

It has been shown that use of different markers may result in very different frequencies even in the same type of cancer due to variable sensitivity of individual markers to detect microsatellite instability [7, 8]. The true incidence and significance of MSI in head and neck cancer is not well established because lack of standardized and comparable parameters among the published studies and a molecular analysis in a large series of patients is lacking. Although a

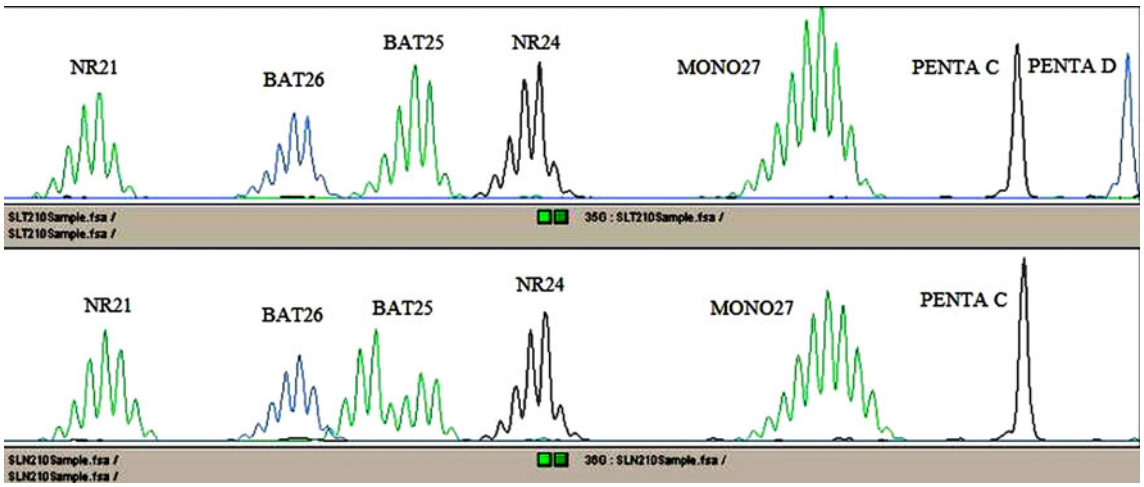


Fig. 1 Microsatellite profile generated using the mononucleotide markers. Instability is observed in the BAT 25 and NR 21 repeats. *Lower panel* tumor tissue, *upper panel* matched normal tissue

few studies have attempted to analyze microsatellite instability in head and neck cancer studies using different sets of microsatellite markers have reported frequencies at great diversity, ranging from 2 to 95% [9, 10]. There are discrepancies due to lack of uniformity in the study methods, choice and number of the markers and criteria required to classify a tumor as having MSI [11, 12]. So far no standard approach for the study of microsatellite instability has been agreed to in head and neck cancer.

New and standardized modern automatic fragment analysis procedures offer clear advantages over previously used gel electrophoresis methods [13] and enable comparison of the results with other cancer types. A reference panel to investigate microsatellite instability consisting of two mononucleotide and three dinucleotide loci has been initially proposed for colon cancer at the Bethesda workshop [14]. Limitations of the original panel have been subsequently modified and revised in the recent years [15]. No significant differences between different cancer types have been reported to justify applying different instability tests for different tumors [16]. The panel used in this study is not tumor specific and has been widely used in different tumor settings.

In this study we analyzed the incidence of microsatellite instability in patients with head and neck cancer using a standardized approach with criteria and markers recommended by NCI.

Table 1 Clinical characteristics of the patients and microsatellite instability

	Total	MSS	MSI-L	MSI-H
Gender				
Male	87	63	8	16
Female	12	10	1	1
Stage				
I, II	24	18	1	5
III, IV	70	52	7	11
Unknown	5	3	1	1
Lymph node status				
(+)	35	28	2	4
(−)	60	42	6	13
Unknown	4	3	1	–
Metastasis				
(+)	16	12	0	13
(−)	81	60	8	4
Unknown	2	1	1	
Histopathology				
Squamous cell ca.	74	55	6	13
Other	19	13	2	4
Unknown	6	5	1	–
Location				
Larynx	65	51	4	10
Other	34	22	5	7

Materials and methods

99 patients with head and neck cancer (mean age 56.8 ± 12.8) were analyzed. Clinopathological characteristics of the patients are shown in Table 1. Fresh tumor and matched normal tissue samples were obtained during surgical resection and stored at −80°C until analysis. The study was approved by the Institutional Ethics Committee. Genomic DNA was extracted from tumor and pathologically confirmed adjacent normal tissue samples by phenol chloroform extraction and proteinase K digestion. Purity of the DNA was determined using the Nanodrop spectrometer (Nanodrop Technologies, Wilmington, USA).

Table 2 Distribution of the positive markers in microsatellite-high and -low samples

	NR 21	BAT 25	BAT 26	NR 24	MONO 27
MSI-H	11	13	6	5	7
MSI-L	–	6	–	1	2

The MSI status of the tumor samples were investigated by a panel of five mononucleotide (NR-21, BAT-26, BAT-25, NR-24 and MONO-27) markers. Two pentanucleotide markers (PENTA C and PENTA D) were used to identify the tumor and matching normal tissue samples.

The PCR reactions were carried out using the Perkin–Elmer GeneAmp 9700 thermal cycler. The reaction mixture contained 2 ng DNA, 1× fluorescently labelled primers, 1× Gold ST buffer, 0.75 U Taq polymerase (AmpliTaq Gold, Applied Biosystems Inc., Foster City, USA) in a total volume of 10 µl. PCR conditions were 95°C for 11 min, 96°C for 1 min followed by 10 cycles of 94°C for 30 s, 58°C for 30 s, 70°C for 1 min and 20 cycles of 90°C for 30 s, 58°C for 30 s and 70°C for 1 min and a final extension at 60°C for 30 min. The amplified products were mixed with deionized formamide and internal standards and analyzed by capillary electrophoresis on the ABI Prism 310 genetic analyzer (Applied Biosystems Inc., Foster City, USA). Internal line standards consisting of 22 DNA fragments labeled with carboxy-X-rhodamine were used to verify amplified sample sizes. The data was evaluated using the GeneScan Software (Applied Biosystems Inc., Foster City, USA). Genomic DNA from K562 cells and nuclease-free water were used as positive- and negative controls, respectively. Correlations between MSI status and clinopathological features were analyzed using the Chi-Square and Fisher's exact tests.

Results

In this study we aimed to determine the frequency of MSI in a large cohort of patients with head and neck cancer using criteria and markers recommended by the NCI and commonly used for different types of cancer. Pentanucleotide markers were included to confirm the identity of matched samples. Presence of unequivocal changes when compared to the matching normal tissue samples (shifts in fragments and new peaks showing up) were classified as microsatellite instability (Fig. 1). Samples displaying instability for two or more markers were considered MSI-H (high) and tumors with changes in one marker only were classified as MSI-L (low). Samples stable for all markers are called microsatellite stable (MSS).

Instability was observed in 26 patients (26%) with 17 patients displaying instability at more than one locus (MSI-H). 73 patients (74%) revealed no differences between tumor and normal tissues and were MSS. Individual markers showed instability in 6–19.2% of the specimens (Table 2). BAT25 and NR21 were the most frequently altered targets. There were only 3 MSI-L samples which did not reveal instability in either of these two loci. In two of these instability was detected at the MONO27 locus and in the remaining sample at the NR24 locus. Instability at the NR21 site was significantly associated with the MSI-H status (Fisher's $P = 0.0016$). Investigation of BAT25 and NR21 loci alone performed as well as the entire pentaplex panel in identifying MSI-H tumors and was found to detect all MSI-H and 88.5% of the MSI-positive samples.

We also investigated the association between the microsatellite instability status and clinicopathologic parameters. There was no significant difference between the MSI-positive and -negative cases in terms of patients age, sex, stage, histopathology, lymph node status and presence of metastases.

Discussion

A variety of molecular and genetic alterations have been described in head and neck cancer. However, there is still a great need for a better understanding of the molecular pathogenesis and for new predictive markers that facilitate early detection or improve classification and treatment outcome in head and neck cancer. It has been suggested that precursor lesions that display microsatellite instability are more prone to progress to carcinomas [17].

In a previous study, using two mononucleotide and one dinucleotide marker we have shown that microsatellite instability and methylation of the mismatch repair genes are frequent in head and neck cancer [6]. However, in absence of established criteria and methodology the data on the prevalence and significance of microsatellite instability in head and neck cancer are still not conclusive and most reports in the literature are prone to be arbitrary [17]. Differences in the study methods and variable sensitivities of the individual markers used do not allow direct comparison of the results.

In this study we aimed to analyze the prevalence of MSI in head and neck cancer using the revised consensus panel consisting only of mononucleotide markers and automated fragment analysis in order to establish a standard and reproducable procedure which will enable comparison of the results between different studies. It has been suggested that the use of five different markers can accurately classify the microsatellite instability status with 100% sensitivity [18]. Five mononucleotide markers were selected because

comprehensive studies investigating hundreds of mono-, di-, tetra-, and penta nucleotide markers have shown that dinucleotide repeats are less sensitive and less specific than mononucleotide repeats [8, 19]. Usefulness of these mononucleotide repeats has been recently confirmed in sporadic endometrial carcinoma [20]. Although mononucleotide repeats are commonly quasimonomorphic obviating the need to test corresponding normal tissue samples [21] we also analyzed matched normal samples from the patients to confirm our results. Moreover, pentanucleotide markers were also included to double-check the identity of these specimens.

We observed microsatellite alterations in 26% of the patients. This value is somewhat lower than our previous report in a similar cohort [6] and results reported for smaller groups of oral [10], sinonasal [22] or double [23] carcinomas but in concordance with recent studies [24–26] all of which involve a much smaller sample size. The discrepancies between studies may be mainly due to the small number of cases involved in the studies, different study methods and the use of different markers. Our data confirm our previous report indicating that MSI is a frequent event in head and neck cancer and are in concordance with methylation of the MMR genes in our patient cohort [6].

Although BAT26 is a sensitive and robust marker for colon cancer [11] allelic changes at this site are less frequent in head and neck cancer. On the other hand, the combination of BAT25 and NR21 was found to identify MSI-H tumors with the same sensitivity and accuracy as the analysis of multiple microsatellite loci. The same finding has been recently reported in a study investigating the performance of different markers [27].

The MSI-L phenomenon is poorly defined and not well understood [28]. It has been previously suggested that MSI-L tumors should be included with MSS cancers for clinical purposes [15]. When MSS and MSI-L tumors are grouped together it is evident that the use of only two markers can detect all MSI samples. Thus this panel of mononucleotide microsatellite repeats can identify MSI-H tumors with high sensitivity and specificity. This is a simple procedure which requires only a single PCR reaction with no need to test normal DNA.

We did not observe any association between the presence of microsatellite instability and clinical parameters. This finding is in accordance with our previous report [6] and several studies in the literature [29–31].

In this study we describe a standardized, rapid and accurate approach to detect MSI simultaneously at different loci. The method allows screening high number of samples with a high specificity. A great advantage of this system is the use of shorter amplified fragments than the Bethesda panel assay which is advantageous for the analysis of formalin fixed, paraffin embedded samples.

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