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REVIEW

The clinical relevance of microsatellite alterations in head and neck squamous cell carcinoma: a critical review

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Triggered by the existing confusion in the field, the current paper aimed to review the current knowledge of both microsatellite instability (MSI) and loss of heterozygosity (LOH) detected by microsatellite markers in head and neck squamous cell carcinoma (HNSCC), and to provide the reader with an assessment of their prognostic and predictive value in this tumor type. For both MSI and LOH, various detection methods were included such as mono- and polynucleotidemarkers and gel- as well as automated analyses. Only studies based on PCR techniques with microsatellite markers were considered. Taking the methodological problems occurring in investigations with microsatellite markers into account, LOH seems to be more common than MSI in HNSCC. Although both types of microsatellite alterations have been correlated with clinicopathological features of this tumor type, only LOH seems to have a clear prognostic value. The predictive value of both MSI and LOH is debatable. More research has to be performed to clearly establish LOH detection as a translational application in the HNSCC field, aiming to predict response to treatments or outcome, and eventually to use as a therapeutic target.
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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common solid tumor worldwide, with an annual incidence of more than 500 000. Traditional treatment of this tumor type is based on combinations of surgery, radiotherapy and chemotherapy with still a rather poor success rate for locally advanced disease. As a result, enormous efforts are currently being put into the search for new molecular markers and associated molecular treatment strategies that might stratify patients and individualize treatment options. With these aims in mind, microsatellite alterations such as microsatellite instability (MSI) and loss of heterozygosity (LOH) gained a lot of interest during the last decade.

The interest in MSI as possible tumor marker was greatly influenced by the marked presence and prognostic value of this phenomenon in Hereditary Non Polyposis Colon Cancer (HNPCC).[1] MSI is also typically associated with Turcot's and Muir–Torre syndrome.[2] However, the frequency and clinical value of MSI in other solid tumors including HNSCC differs widely, largely due to an obvious variance in used methods and criteria.[2]

Concerning LOH, different techniques have been developed to assess this type of alterations. The further

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report on LOH in this work will only refer to LOH detected with microsatellite markers. Problems of methodological variance trouble the reported frequencies, in alignment with MSI assessment. The prognostic and predictive value of this phenomenon varies along different tumor types (bladder, prostate, brain cancer).[3–5] Although the potential of LOH as a molecular prognostic marker in HNSCC has gained confidence over the years, literature on this field stays confusing.

The aim of this paper was to review the current knowledge of microsatellite alterations, both MSI and LOH, in HNSCC and to provide the reader with an assessment of their prognostic and predictive value.

What are MSI and LOH?

Microsatellites are stretches of DNA in which a short motif (usually 1–5 nucleotides long) is repeated 5–100 times. Microsatellite regions are at high risk for variations in the number of repeats caused by slippage of the DNA polymerase during DNA replication. In normal cells, these errors get repaired by the mismatch repair system (MMR) involving proteins such as MLH1, MLH3, MSH2, MSH3, MSH6, PMS1 and PMS2. In tumors, defects in the MMR system may be present, so that variations in microsatellite regions are not repaired correctly, leading to definitive somatic changes with gain or loss of repeat units. MSI can thus indicate the presence of a defect MMR system. Interestingly, MMR deficiency can lead to carcinogenesis as a result of mutations in key genes, defining a so-called mutator phenotype. According to Field, MSI could serve as an indirect marker for the somatic mutations caused by the mutator phenotype.[6] This mutator phenotype is an important carcinogenetic mechanism in HNPCC, which is associated with inherited MMR mutations.[1]

Genetic and epigenetic inactivation of the MMR pathway is however not a commonly event in solid tumor types other than HNPCC. Therefore, MSI in these other tumor types, if observed, may be due to factors other than defects in MMR genes such as malfunctioning of factors downstream of MMR components or in other systems that proofread DNA for replication errors.[7] Although not clearly established, environmental factors such as oxygen radicals, lipid adducts, smoking or diet also could play a role in generating MSI. These exposures can act alone or in concert with defective DNA repair pathways.[8]

LOH, in contrast, marks a suppressor phenotype that is characterized by wide variety in chromosomal numbers (aneuploidy) and extensive loss of genetic material. Even if nowadays most LOH investigations use comparitive genomic hybridization or single-nucleotide polymorphisms (SNPs) techniques, the presence of LOH can also be detected with the use of microsatellite markers. Accordingly, LOH can be recognized by loss of a genomic fragment, SNP or microsatellite allele in a tumor when compared with normal tissue. LOH is a common mechanism of inactivation of tumor suppressor genes (TSG) located in the neighborhood of the allelic marker that is being detected. TSG loss is normally confirmed by investigating gene deletion or mutation, silencing by DNA methylation or concurrent loss of protein expression.[9]

Prevalence of MSI and LOH in HSNCC

MSI

In HNSCC, the reported frequencies of MSI vary widely but in general the incidence is low leaving any significance unclear. Table 1 that shows the published studies till now on MSI in HNSCC indicates that the incidence varies from 3[21] to 88%[24] of MSI-H, largely depending on the number of patients, the loci and markers used, the patient age and detection methods.

In general, MSI in HNSCC seems to be a late event associated with tumor progression. Most studies show that invasive carcinomas manifest more MSI than precursor lesions, suggesting progressive accumulation of MSI during tumor development[12,28] (Table 1). Furthermore, precursor lesions that show MSI are more prone to progress to HNSCC.[19] Arguments against this vision arise from studies showing high MSI incidences in pre-malignant lesions and in young patients.[20,24]

Most studies report on a lack of MMR gene defects in HNSCC compared with HNPCC.[14,21,24,29] This could either indicate a low incidence of MSI or a higher proportion of non-MMR MSI cases. In HNSCC, this could be caused by common carcinogens for this patient group, such as smoking. However, it could also mean that 'true' MSI in HNSCC does not exist – at least not in the way it does in HNPCC.

LOH

Numerous different techniques have been used to assess LOH alterations in HNSCC. Perhaps this is one reason why, although the potential of this molecular prognostic marker has gained confidence over the years, the literature in this field stays confusing. Table 2 lists the published studies on LOH detected with microsatellite markers in HNSCC.

Most suggest that the frequency of LOH is higher than of MSI, indicating that the suppressor phenotype could be more prevalent.[11,12,36] LOH and the spectrum of chromosomal loss progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma *in situ* to invasive cancer.[19,33,53] Some primary and recurrent HNSCC originate from the same precursor lesion that contains genetically related features to the tumors, including LOH.[54]

Overall, studies in HNSCC have shown that deletions at chromosome arms 3p, 4p, 8p and 9p represent early

Table 1 MSI in HNSCC

Number of patients	*Chromosomal arms or chromosomes*	*Markers (n)*	*MSI (%)*	*Reference*
40	3p	10	15% MSI	Arai *et al*[10]
56 primary, 23 resistant to chemo	3p, 9p, 17p, 8p, 18q	22	2% MSI-H 11% MSI-L	Blons *et al*[11]
20 (normal mucosa, dysplasia and carcinoma of same patients)	3p, 5p, 5q, 8p, 9p, 9q, 11q, 17p, 17q, 18p, 18q	25	15% dysplasia MSI+ 30% invasive ca MSI+	El Naggar *et al*[12]
38	1p, 3p, 3q, 4q, 5p, 6p, 7, 8p, 9p, 11q, 13q, 14q, 17p, 18q	28	13% MSI in one marker	Fiedler *et al*[13]
56	2, 4, 6, 7, 8, 10, 13, 14, 16, 17	34	28% MSI $\geq$2 markers	Field *et al*[6]
153	1p, 2p, 3p, 4q, 5q, 9p, 9q, 11q, 17p, 17q, 18q	22	14% MSI 3% MSI-H 1% MSI at BAT 25	Glavac *et al*[14]
23	2p, 1p, 1pter-1qter, 3p, 4q, 5q, 7q, 8pter-8qter	9	Colon cancer history: 25% MSI-H, 25% MSI-L No colon cancer history: 20% MSI-L, no MSI-H	Gleich *et al*[15]
91	3p, 6p, 7q, 9p, 11p, 11q	19	7% MSI	Ishwad *et al*[16]
35	X, 14, 19, 6, 12, 4, 21	52	29% MSI at $\geq$1 locus	Mao *et al*[17]
30	3p, 4q, 7p, 9p, 17p, 18q	17	13% MSI (at 1 locus)	Ng *et al*[18]
39 HNSCC patients with history of dysplasia, 39 controls with dysplasia but no HNSCC	3p, 4q, 5q, 8p, 9p, 13q, 17p, 18q	25	Cases: 18% MSI-H, 23% MSI-L Controls: 0% MSI-H, 15% MSI-L	Partridge *et al*[19]
31 pre-malignant lesions	3p21, 8p21-23, 9p21 (1q, 3q, 7q, 19q, 20q as control markers)	23	55% MSI at $\geq$1 locus	Partridge *et al*[20]
67	1p, 3p, 3q, 9p, 13q, 19p	20	4% MSI-L, 3% MSI-H	Piccinin *et al*[21]
46	3p24-pter	6	16% MSI 57% MSI or LOH	Rowley *et al*[22]
41	18	10	17% MSI 56% MSI or LOH	Rowley *et al*[23]
24 $\leq$44 years 33$\geq$45 years	2p, 3p, 5q, 8p, 9p, 13q, 14q, 14pter, 17q, 21q	16	$\leq$44 years: 100% MSI at $\geq$1 loci 88% MSI at $\geq$2 loci $\geq$45 years: 61% MSI at $\geq$1 site 36% at $\geq$2 sites	Wang *et al*[24]
32 oral SCC	8p	14	50% MSI	Ono *et al*[25]
41 oral SCC	1p	15	44% MSI	Araki *et al*[26]
52	3p, 9p, 17p	35	46% MSI at $>$2 loci	Nunn *et al*[27]
111 hyperplasia to invasive	3p, 9p, 17p, 5q	6	14% MSI at $\geq$1 loci	Ha *et al*[28]

This table represents published studies on MSI showing the number of patients included, the chromosomal arms or chromosomes examined, the number of microsatellite markers used and the reported percentage of MSI.

genetic changes and that loss at 18q, 17p and 11qter is associated with neoplastic progression.[33,34,36,45,48,55] Dysplastic regions in the head and neck region that show more LOH seem to be more at risk for neoplastic evolution.[17,18,19,33]

There are several reports that LOH is associated with more advanced stage and more aggressive HNSCC tumors,[13,34,36,37,56] and specifically with nodal involvement.[32,44] Other authors describe very specific correlations such as a link between LOH at 17p and mitotic index,[39] a connection of LOH at MLH1 with lower grade HNSCC, and LOH at CDKN2A with higher grade,[9] or a preference of LOH in tumors originating from the pharynx.[56] A correlation between LOH and other patient-related factors like age and race, was described in some studies.[34,51]

Methodological issues

The first critical point in MSI and LOH detection is microsatellite marker selection. The use of the Bethesda MSI reference set[8] that was developed for HNPCC *versus* presumably more HNSCC-selective markers is hotly debated, as is the use of mononucleotide *versus* polynucleotide markers.[17,57–59] However, most researchers currently accept that for MSI assessment, quasimonomorphic mononucleotide markers are suitable. For the detection of LOH with microsatellite markers, locus selection is dependent on the specific chromosomal region and possible associated TSG that the researchers reckon to be relevant.

Second cause for the observed variance in MSI and LOH reports lies in the use of different detection methods. The modern automatic fragment analysis procedures offer high throughput analysis and a more precise and quantitative

Table 2 LOH in HNSCC

Number of patients	*Chromosomal arm or chromosomes*	*Markers (n)*	*Conclusion*	*Reference*
28	All limbs, except 13p, 14p, 15p, 21p, 22p	50	High LOH incidence at 3p, 5q, 9q, 11q, 17p	Ah-See *et al*[30]
40	3p	10	Most LOH near VHL locus	Arai *et al*[10]
64	9p	1	26% LOH	Bazan *et al*[81]
56 primary, 23 resistant	3p, 9p, 17p, 8p, 18q	22	75% LOH	Blons *et al*[11]
48 primary, 8 M+	10q	4	10q25-q26 highest LOH%	Bockmuhl *et al*[32]
83 (preinvasive)	9p, 11q, 17p, 3p, 13q, 4q, 14, 8p, 6p	26	Most LOH at 9p, 3p, 11q	Califano *et al*[33]
92 (48 variants and 44 conventional)	3p, 4p, 8p, 9p, 11q, 17p, 18q	21	Limited set of markers (at 4p, 9p, 11q and 18q) identifies most LOH	Choi *et al*[34]
150	3p, 5q, 8p, 9p, 10p, 18q, 21q	26	LOH at 3p:67%, at 8p: 43%, at 9p: 61%.	Coon *et al*[35]
20 (normal mucosa, dyplasia and tumor of same patients)	3p, 5q, 8p, 9p, 9q, 11q, 17p, 3p, 18p	25	LOH high at 9p, 8p, 3p, 9q and 11q	El Naggar *et al*[36]
38	1p, 3p, 3q, 4q, 5p, 6p, 7, 8p, 9p, 11q, 13q, 14q, 17p, 18q	28	Most LOH on 3p, 9p and 13q	Fiedler *et al*[13]
80	All autosomal chromosome arms except 13p, 14p, 15p, 21p, 22p	145	Highest LOH on 3p, 9p, 17p and 18q	Field *et al*[37]
153	1p, 2p, 3p, 4q, 5q, 9p, 9q, 11q, 17p, 17q, 18q	22	78% LOH Most LOH in 3p14-p26, 9p21, 17p12-p13	Glavac *et al*[14]
23	2p, 1p, 1pter-1qter, 3p, 4q, 5q, 7q, 8pter-8qter	9	More LOH in the group with colon cancer history	Gleich *et al*[15]
43	3p	23	81% LOH at one or more 3p markers 66% LOH at 3p21.3 56% LOH at 3p12	Hogg *et al*[31]
77	3p, 9p, 6p, 7q, 11p, 11q	16	71% LOH at $\geq$1 loci 58% LOH at 3p 48% LOH at 9p	Ishwad *et al*[38]
32 hyperplastic pre-malignant laryngeal lesions	3p, 6p, 6q, 8p, 9p, 9q, 13q, 17p, 18q	32	Most LOH at 3p21-14 and 9p22-21	Kleist and Poetsch[39]
26 dysplastic epithelium	9p21	3	35%-82% LOH	Kresty *et al*[40]
68	1p, 1q, 2p, 3p, 4p, 4q, 5p, 5q, 6p, 7p, 7q, 8p, 9p, 10p, 10q, 11q, 12p, 13q, 14q, 15q, 16p, 16q, 17q, 18p, 19p, 19q, 20p, 20q, 21q, 22q	43	Most ($>$20%) LOH at 3p21, 3p25-26, 8pter-21.1, 13q14, 17p12	Li *et al*[41]
48	13q	11	67% LOH at 13q, mostly loss of entire chromosomal arm	Maestro *et al*[42]
29	All somatic chromosome arms, except 13p, 14p, 15p, 21p, 22p	58	72% LOH at 9p 50% LOH at 3, 11q, 13q, 17p $>$35% at 4, 6p, 8, 14q, 19q	Nawroz *et al*[43]
30	3p, 4q, 7p, 9p, 17p, 18q	17	30% LOH, most at 7q and 9p	Ng *et al*[18]
34	13q14.3	18	68% LOH	Ogawara *et al*[44]
39 HNSCC patients with history of dysplasia, 39 controls with dysplasia but no HNSCC	3p, 4q, 5q, 8p, 9p, 13q, 17p, 18q	25	Most LOH at 3p21, 9p21, 13q14.2, 17p13.1, 18q21.1	Partridge *et al*[19]
48 oral SCC	3p	15	71% LOH at $\geq$1 locus	Partridge *et al*[45]
31 pre-malignant lesions	3p21, 8p21-23, 9p21 (1q, 3q, 7q, 19q, 20q as control markers)	23	77% AI (LOH and allelic gain)	Partridge *et al*[20]
67	1p, 3p, 3q, 9p, 13q, 19p	20	7-77% LOH, most at 3p and 13q	Piccinin *et al*[21]
46	3p24-pter	6	48% LOH at $\geq$1 locus 57% LOH or MSI Most LOH between D3S656 and D3S1293	Rowley *et al*[22]
41	18	10	51% LOH, most 18q	Ono *et al*[25]
62 larynx	3p, 8p, 13q, 9p, 1q, 5q, 2p, 17p, 17q, 21q	13	Most LOH at 9p, 3p, 8p, 13q, 2p	Sasiadek *et al*[9]
59 larynx	8p	3	LOH of D8S264 independent negative prognostic factor[a]	Scholnick *et al*[46]
31 oral cancer	11	22	56% LOH at $\geq$1 locus, most at 11q	Uzawa *et al*[47]

Table 2 (*Continued*)

Number of patients	*Chromosomal arm or chromosomes*	*Markers (n)*	*Conclusion*	*Reference*
29 invasive, 17 preinvasive	9	15	72% LOH in invasive 71% in preinvasive	van der Riet *et al*[48]
26	3p	4	100% LOH at $\geq$1 marker surrounding VHL locus	Waber *et al*[49]
21	9p21-22	8	81% LOH at $\geq$1 marker	Waber *et al*[50]
18 African Americans (AA), 19 Non-African Americans (NAA)	1p, 3p, 4q, 9p, 13q, 17p	18	68.8–83.3% LOH in AA 66.7–90.0% LOH in NAA	Yoo *et al*[51]
32 oral SCC	8p	14	62.5% LOH at $\geq$1 locus	Ono *et al*[25]
41 oral SCC	1p	15	73% LOH at $\geq$1 locus	Araki *et al*[26]
52	3p, 9p, 17p	35	69% LOH at 17p 64% LOH at 3p 61% LOH at 9p	Nunn *et al*[27]
59 laryngeal SCC	8p	17	39% LOH at $\geq$1 locus	Sunwoo *et al*[52]

AI, allelic imbalance.
This table represents published studies on LOH showing the number of patients included, the chromosomal arms or chromosomes examined, the number of microsatellite markers used and the most important conclusions, respectively.
[a]No information on LOH prevalence in this study population.

assessment of MSI and LOH in comparison to the previously used gel electrophoresis methods.[60–64] Although other points of discussion are the cutoff values to be used for LOH ratios (the ratio between the peak height ratios of both alleles of normal and paired tumor sample),[9,39,63,65] there is fairly general agreement that appropriate cutoffs are >2 or <0.5. A last issue is on the different types of MSI. Most investigators discern MSI-High (MSI-H, MSI in $\geq$30–40% of the markers tested) and MSI-Low (MSI-L, MSI in $<$30–40% of the markers tested),[8] although others call these Type I and II[62] or Type B and A instability,[66] respectively.

Prognostic and predictive value of MSI and LOH in HNSCC

MSI

Prognostic value In HNSCC, the prognostic impact of MSI is not clear.[2] Some studies showed that MSI positivity in pathologically negative surgical margins of HNSCC can independently predict local recurrence.[67,68] Some authors found no correlation between MSI in HNSCC and survival,[6] whereas others describe a better prognosis for MSI-positive patients,[69] as recently also published for colorectal cancer.[1] A possible explanation for the lack of prognostic value is the lack of statistical power in a lot of studies owing to the overall low prevalence of MSI in HNSCC, next to the previously mentioned difference in methodology.

Predictive value As MMR-proficient cells undergo a cytotoxic reaction after recognition of drug-induced adducts in DNA, MMR deficiency can theoretically impart resistance to cancer chemotherapy agents. Some mainly *in vitro* and animal work on different tumor types indeed suggested that MMR-deficient cells have a poorer response to cisplatin, carboplatin and methylating agents.[7,69–71] However, clinical studies in colorectal cancer, in which a relation between MSI and MMR deficiency is clearly established, have not shown a consistent predictive value of MSI.[1,72] For ionizing radiation (IR), a link with MMR defects is unlikely, as IR mainly causes DNA strand breaks and repair of this damage does not involve the MMR system. Therefore, it is not surprising that no predictive value of MSI towards chemotherapy or radiotherapy has been found in HNSCC, a malignancy in which the occurrence of MMR deficiency is uncertain.[11]

LOH

Prognostic value In general, LOH at multiple loci is associated with a poor clinical course such as nodal invasion or high-grade disease.[44,55] Furthermore, several studies have identified a negative prognostic role for LOH,[18,37,41,73,74] even in multivariate analyses.[34,35,46] This negative prognostic value is mostly connected to allelic loss at 3p, 8p, 11q, 13q, 17p and 18q. On the other hand, LOH at 9p21 seems to be an early event in carcinogenesis (like LOH at 3p) that has not consistently been connected with a poor prognosis,[34,35,75] but along with LOH at 7q31 has been related to tumor recurrence.[76,77] At the most common loci, the occurrence of LOH is suggested to be associated with loss of TSG: FHIT at 3p, $p16^{INK4A}$ at 9p, Rb at 13q and so on. For all of these TSG, a prognostic role has been described in HNSCC.[78–80]

Predictive value One study showed that microsatellite alterations in a histopathologically negative surgical margin can predict local recurrence.[68]

LOH is a frequent mechanism of inactivation of TSG, which might be involved in resistance to chemotherapy. A study in HNSCC showed an association between LOH at 9p or 17p resistance to a chemotherapy regimen consisting of cisplatin and 5-fluorouracil, with an independent

negative predictive role for LOH at the p53 locus on 17p. The authors concluded that p53 alterations could play a role in chemotherapy resistance in HNSCC.[11]

Conclusion

This review aimed to explore current knowledge about microsatellite alterations (MSI/LOH) in HNSCC. HNSCC typically evolves from normal epithelium through dysplasia, carcinoma *in situ* finally to the invasive carcinoma stage. During this tumorigenesis, cumulative genetic alterations including MSI and LOH occur.

In HNSCC, most recent research efforts have been put into the investigation of LOH at several chromosomal loci. These alterations, representing the suppressor phenotype, seem to be more common than MSI in HNSCC. Although both types of microsatellite alterations have been correlated with clinicopathological features of head and neck cancer, only LOH seems to have a clear prognostic value. The predictive value of both MSI and LOH towards surgery, radiotherapy and chemotherapy is debatable. Biggest challenges however remain in the methodological problems connected with these types of investigations.

We recently tried to determine the real MSI and LOH prevalence in HNSCC, using automatic fragment analysis as the preferred technique to assess MSI and LOH, several panels of microsatellite markers in an attempt to compare their sensitivity, and strict cutoff values for LOH detection. This study resulted in a very low (around 1%) percentage of MSI, suggesting that indeed the prevalence of MSI in HNSCC has been overestimated in literature, partly due to the use of non-optimal techniques (De Schutter *et al*, submitted).[82] Based on this experience, a role for MSI as prognostic or predictive marker in this tumor type seems highly unlikely.

On the other hand, the detection of LOH with the use of microsatellite markers in HNSCC seems feasible and of clinical importance. As LOH at certain loci may be indicative for the loss of a TSG, therapeutic options would mainly be directed towards re-expression of the involved gene, which is the goal of several gene therapy trials. However, re-expression therapies are mainly experimental and still face a lot of difficulties.

More research has to be performed to establish clearly LOH detection as a translational application in the HNSCC field, aiming to predict response to treatments or outcome, and eventually to use as a therapeutic target.

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