

Keywords: Tetranucleotide repeats; microsatellite repeats; prevalence; microsatellite instability; DNA mismatch repair; solid tumours; TP53; MSH3; T cells; cancer early detection; prognosis

Prevalence and implications of elevated microsatellite alterations at selected tetranucleotides in cancer

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Elevated microsatellite alterations at selected tetranucleotides (EMAST), a variation of microsatellite instability (MSI), has been reported in a variety of malignancies (e.g., neoplasias of the lung, head and neck, colorectal region, skin, urinary tract and reproductive organs). EMAST is more prominent at organ sites with potential external exposure to carcinogens (e.g., head, neck, lung, urinary bladder and colon), although the specific molecular mechanisms leading to EMAST remain elusive. Because it is often associated with advanced stages of malignancy, EMAST may be a consequence of rapid cell proliferation and increased mutagenesis. Moreover, defects in DNA mismatch repair enzyme complexes, TP53 mutation status and peritumoural inflammation involving T cells have been described in EMAST tumours. At various tumour sites, EMAST and high-frequency MSI share no clinicopathological features or molecular mechanisms, suggesting their existence as separate entities. Thus EMAST should be explored, because its presence in human cells may reflect both increased risk and the potential for early detection. In particular, the potential use of EMAST in prognosis and prediction may yield novel types of therapeutic intervention, particularly those involving the immune system. This review will summarise the current information concerning EMAST in cancer to highlight the knowledge gaps that require further research.

Microsatellites are repeating units of one to six base pairs, which are ubiquitous, abundant and repeated several times in eukaryotic genomes. These repeats are prone to errors during DNA replication, and the failure to correct such errors results in microsatellite instability (MSI). Since its initial description as a form of genetic instability in colorectal cancer (CRC), MSI has been linked to a number of phenotypic characteristics and clinicopathological features of tumours (Sinicrope and Sargent, 2012). The prognostic and predictive relevance of MSI in CRC has been well documented (Sinicrope and Sargent, 2012). In CRC, MSI is the hallmark of forms of hereditary non-polyposis CRC (HNPCC; or Lynch syndrome) caused by germline mutations in mismatch repair (MMR) genes. MSI is also detected in 15% of sporadic CRC cases, usually as a consequence of epigenetically silenced MMRs (Sinicrope and Sargent, 2012). Furthermore, the

presence of MSI has been demonstrated in other cancers, including endometrial, ovarian and urinary tract cancers. Currently, MSI appears to be a genetic aberration found in a wide range of human solid tumours that is best defined in the context of colorectal neoplasia; at tumour sites other than CRC, the clinical relevance and frequency of MSI are variable (Catto *et al*, 2003; Pal *et al*, 2008; Diaz-Padilla *et al*, 2013). In the clinical setting, such as when screening for CRC, HNPCC or sporadic MSI, MSI is usually classified as mononucleotide and dinucleotide repeats (Umar *et al*, 2004). Recently, a number of reports have also documented instability at specific tetranucleotide repeats, a phenomenon termed elevated microsatellite alterations at selected tetranucleotide repeats (EMAST). As an emerging specific form of genetic alteration, EMAST remains poorly described. However, EMAST may hold clues for deeper insights into the roles of MSI in cancer

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Revised 1 March 2014; accepted 5 March 2014; published online 1 April 2014

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biology, thus facilitating improved preventive, therapeutic and prognostic/predictive strategies in human cancer. This review aims to concisely present the current knowledge concerning EMAST in human solid cancers; we briefly present the proposed molecular mechanisms and describe the potential clinical implications for human cancer.

MICROSATELLITES, REPEAT SIZE AND INSTABILITY

During DNA replication, microsatellites are prone to erroneous duplication, which involves the addition or deletion of one or several repeated units, and the failure of DNA proofreading mechanisms to correct such errors results in instability at microsatellites. Microsatellite alterations, such as the insertion/deletion of a repeat, can produce deleterious effects via the induction of strand slippage and frameshift mutations when a protein-coding region is affected (Saeterdal *et al*, 2001; Kim *et al*, 2013). The results of such effects range from changes in the physical and chemical properties of the translated proteins to interference with transcription at promoter sites or the translation of truncated and dysfunctional proteins.

Generally, repetitive elements can occur in a variety of patterns and distributions (i.e., tandem, dispersed and paralogues). These elements constitute as much as 50% of the genome, and approximately one-fourth of empirically defined human promoters are surrounded by or contain clustered repetitive elements. However, the genomic distribution of microsatellites is rather random; mononucleotides, dinucleotides and tetranucleotides are primarily found in intronic and intergenic noncoding regions, whereas other microsatellites (trinucleotides and hexanucleotides) are located more abundantly within sequences involved in gene transcription (Subramanian *et al*, 2003).

Previous attempts at defining MSI have not produced straightforward results. Following the first MSI international workshop at the National Cancer Institute (NCI) at Bethesda in 1998, the 2004 revised NCI/Bethesda microsatellite screening panel (Umar *et al*, 2004), which is based on five mononucleotide and dinucleotide microsatellites, defined 'classical' MSI as high-frequency (MSI-H; ≥ 2 out of 5 markers are unstable or $\geq 40\%$), low-frequency (MSI-L; 1 out of 5 markers is unstable or $\geq 20\%$ and $< 40\%$) or microsatellite-stable (MSS; no unstable markers detected). In CRC, there is a consensus on the definitions of MSI for clinical use (Umar *et al*, 2004), although defining the different degrees of instability remains a matter of controversy. Based on the Bethesda panel, MSS, MSI-L and MSI-H tumours contain different numbers of altered microsatellites, suggesting that these forms of MSI represent separate phenomena, at least at the molecular level. However, MSS tumours and MSI-L unstable tumours are typically grouped together, because both present similar clinical forms and gross abnormalities. Notably, MSI-L is generally detected only when dinucleotide markers are used, and some authors have disputed the existence of MSI-L completely (Kim *et al*, 2013). MSI is found with the highest frequency in mononucleotide repeats, which are also the most frequently occurring nucleotide repeats throughout the genome. Notably, tetranucleotide repeats occur at a much lower frequency than mononucleotide repeats, and it is not known whether forms of MSI involving nucleotide repeats of different sizes share common underlying mechanisms. However, the most clinically relevant microsatellites studied in cancer are composed of mononucleotide (e.g., G_n), dinucleotide (e.g., CA_n) or tetranucleotide (e.g., AAAG_n) repeats. Variations in the number of repeats in microsatellite sequences among the population are defined as microsatellite polymorphisms, as opposed to MSI, which refers to such alterations within multiple cells of the same individual (e.g., when comparing tumour and normal tissues). Instability at tetranucleotides represents a variation of MSI that

was not addressed by the Bethesda definitions (Umar *et al*, 2004), which are based on mononucleotide and dinucleotide markers. However, in most EMAST-associated cancers, instability occurs at loci containing AAAG_n or ATAG_n repeats.

PREVALENCE AND RELEVANCE OF EMAST IN HUMAN CANCERS

EMAST has been reported in several cancers of solid organs (Figure 1), including the colorectum (Haugen *et al*, 2008; Devaraj *et al*, 2010; Lee *et al*, 2010; Lee *et al*, 2012), lungs (Ahrendt *et al*, 2000; Xu *et al*, 2001; Arai *et al*, 2013), ovaries (Singer *et al*, 2004), bladder (Xu *et al*, 2001; Danaee *et al*, 2002; Catto *et al*, 2003; Burger *et al*, 2006a), prostate (Perinchery *et al*, 2000; Burger *et al*, 2006b; Azzouzi *et al*, 2007), kidney (Xu *et al*, 2001; Catto *et al*, 2003), head and neck (Xu *et al*, 2001; Temam *et al*, 2004), non-melanoma skin (Danaee *et al*, 2002) and uterus (Choi *et al*, 2008). The estimated prevalence of EMAST according to previously reported studies is presented in Table 1. In fact, the reported prevalence and relationship between EMAST and clinicopathological features and molecular mechanisms vary considerably across tumour sites (Figure 1).

CRCs. Presumably because of the great impact that MSI has had on the understanding of human cancer biology, the vast majority of EMAST-related reports in the scientific literature concern CRC. EMAST is found most frequently ($> 60\%$) in advanced-stage CRC with poor tumour differentiation (Lee *et al*, 2010). The documented presence of EMAST in adenomas has been inconsistent, with reports ranging from 0% (Lee *et al*, 2012) to 33% (Lee *et al*, 2010). The overall frequency of EMAST in CRC is much higher (up to 60%) than the frequency of classical MSI-H (usually 15–20%) (Yamada *et al*, 2010), and EMAST occurs at high rates in rectal cancers (Devaraj *et al*, 2010). EMAST is associated with the MSI-L classification and demonstrates a worse prognosis with a shorter time to recurrence and development of distant metastasis in stage II and III CRC (Garcia *et al*, 2012). This worse prognosis is observed regardless of the association of EMAST with greater infiltration of CD8+ T cells in both the tumour and surrounding stroma (Devaraj *et al*, 2010; Lee *et al*, 2012), a feature usually associated with better prognosis in CRC. Thus the dual role of inflammation in this setting warrants further investigation.

Respiratory tract cancers. The reported frequency of EMAST in lung cancers ranges from 32% to 64.5% (Ahrendt *et al*, 2000; Xu *et al*, 2001; Woenckhaus *et al*, 2003; Arai *et al*, 2013). However, there appears to be a distinction between EMAST and MSI-H, and mutations in *TP53* are common in EMAST cancers (Ahrendt *et al*, 2000; Xu *et al*, 2001; Woenckhaus *et al*, 2003). EMAST was found to be most prevalent in cases of the squamous subtype and early stage disease (Xu *et al*, 2001; Woenckhaus *et al*, 2003; Arai *et al*, 2013), although it was associated with higher rates of lymph node metastasis in another study (Woenckhaus *et al*, 2003). Additionally, one study found a higher incidence of previous cancer history in patients with EMAST (Arai *et al*, 2013), potentially suggesting a 'field defect' or cancer susceptibility related to EMAST.

Kidney and urinary tract cancers. The frequency of EMAST in cancers of urogenital organs is markedly lower compared with that in other solid tumours and is higher in the lower urinary tract (urinary bladder) than the upper urinary tract (kidneys, ureters and renal pelvis) (see Table 1, Figure 1).

In bladder cancer, EMAST-positive tumours (approximately one-third of bladder tumours) have been associated with *TP53* mutations and non-invasive lesions (Danaee *et al*, 2002). However, a much lower EMAST frequency (5.3%) was demonstrated in a large, non-selected cohort of urothelial bladder cancers, and

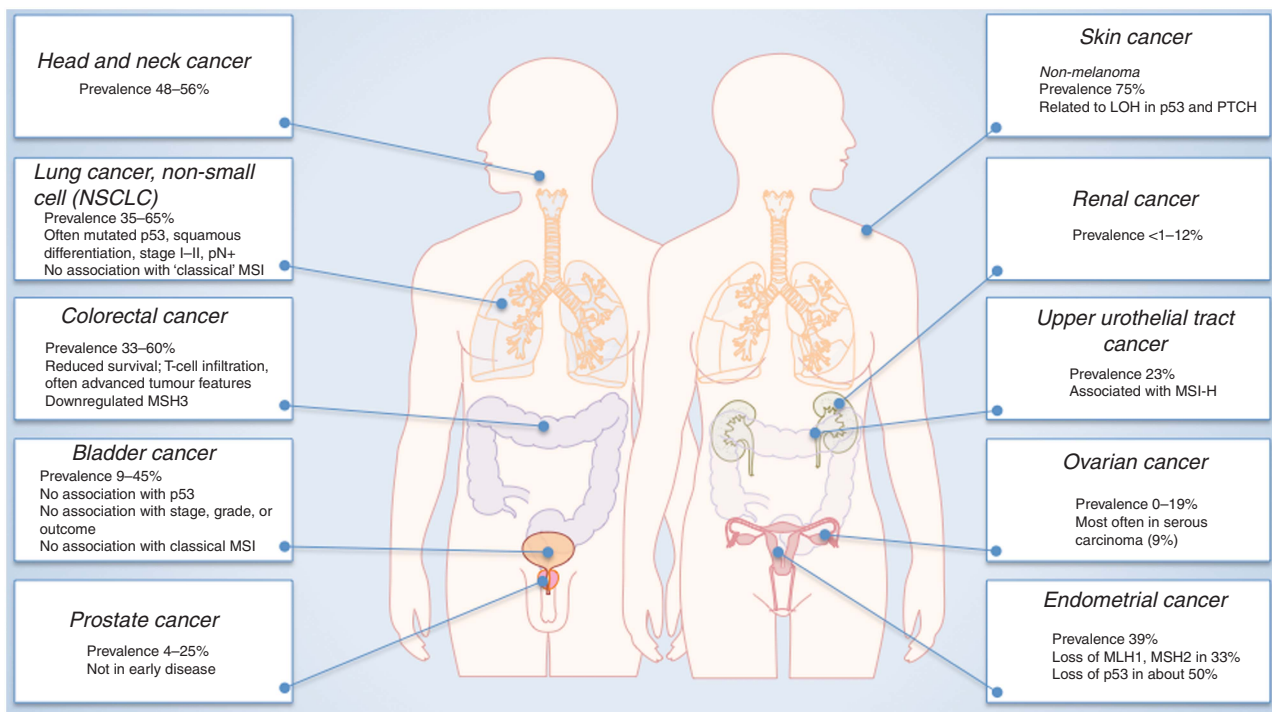


Figure 1. Prevalence and main observed features of EMAST in human solid cancers.

Table 1. Prevalence of EMAST among human solid malignancies

Site	Author, year	Cohort size (n)	Definitions	Prevalence (%)
Bladder	Xu <i>et al</i> , 2001	38	≥ 1/12 markers unstable	21%
	Burger <i>et al</i> , 2006a, b	117	≥ 1/10 markers unstable	9%
	Catto <i>et al</i> , 2003	89	≥ 1/8 markers unstable	45%
	Danaee <i>et al</i> , 2002	57	≥ 1/7 markers unstable	44%
Colorectal	Lee <i>et al</i> , 2010	108	≥ 2/5 markers	50%
	Lee <i>et al</i> , 2012	108	≥ 2/5 markers	50%
	Devaraj <i>et al</i> , 2010	147 (only rectal)	≥ 2/5 markers	33%
	Haugen <i>et al</i> , 2008	117	≥ 1/7 markers unstable	60%
NSCLC	Xu <i>et al</i> , 2001	47	≥ 1/12 markers unstable	51%
	Arai <i>et al</i> , 2013	65	≥ 1/10 markers unstable	65%
	Ahrendt <i>et al</i> , 2000	88	≥ 1/13 markers unstable	35%
Prostate	Burger <i>et al</i> , 2006a, b	81	≥ 1/10 markers unstable	5%
	Azzouzi <i>et al</i> , 2007	50	≥ 2/4 markers	4%
	Perinchery <i>et al</i> , 2000	40	≥ 1/4 markers	25%
Renal	Xu <i>et al</i> , 2001	25	≥ 1/12 markers unstable	12%
	Catto <i>et al</i> , 2003	71 (upper urinary tract)	≥ 1/8 markers unstable	23%
Head and neck	Xu <i>et al</i> , 2001	18	≥ 1/12 markers unstable	56%
	Temam <i>et al</i> , 2004	54	≥ 1/5 markers	48%
Non-melanoma skin	Danaee <i>et al</i> , 2002	61	≥ 1/7 markers unstable	75%
Ovarian	Singer <i>et al</i> , 2004	53	≥ 1/6 markers unstable	19%
Endometrial	Choi <i>et al</i> , 2008	39	≥ 1/6 markers unstable and no instability in mononucleotides and dinucleotides	39%

Abbreviation: NSCLC = non-small cell lung cancer.

EMAST was not found to be associated with TP53 status (Burger *et al*, 2006a). In the urinary tract, EMAST appears to be unrelated to classical MSI and occurs less frequently in the renal pelvis (Catto *et al*, 2003). No firm associations between EMAST and clinicopathological features or clinical outcomes have been reported (Danaee *et al*, 2002; Catto *et al*, 2003; Burger *et al*, 2006a). The reported EMAST frequency in renal cell carcinomas is very low, ranging from <1% (Stoehr *et al*, 2012) to 12% (Xu *et al*,

2001). Based on the limited available evidence, it appears that the prevalence of EMAST decreases from the upper to the lower urinary tract, with no solid clinical relationships or molecular mechanisms having been established in this area.

Reproductive organs. In cancers of the reproductive organs, EMAST occurs slightly more often in malignancies of the female reproductive organs, such as the ovaries (13%, Singer *et al*, 2004)

and uterus (38.5%, Choi *et al*, 2008), compared with the male reproductive organs, although the overall available literature remains limited.

Classical MSI is noted in approximately 12% of ovarian cancers (Pal *et al*, 2008) and is most frequently associated with the non-serous histological type. In contrast, EMAST appears predominantly in the serous subtype of ovarian cancers; no EMAST has been found in non-serous carcinomas (Singer *et al*, 2004).

Approximately 15% of endometrial cancers exhibit MSI-H, but there are conflicting reports of the relation to clinical outcome (Diaz-Padilla *et al*, 2013). EMAST has been reported at a higher frequency (39%) than classical MSI, but these two markers showed no correlation in type I endometrial carcinomas. Furthermore, most (70%) EMAST-positive tumours exhibited normal expression of both MSH2 and MLH1 in immunohistochemical analyses (Choi *et al*, 2008).

In prostate cancer, MSI is generally prevalent at dinucleotide tandem repeats and is less common in trinucleotide and tetranucleotide repeats (Perinchery *et al*, 2000). According to most reports, the frequency of EMAST in prostate cancer is <5% (Terrell *et al*, 1995; Burger *et al*, 2006b; Azzouzi *et al*, 2007). In prostate cancer, EMAST is related neither to MSI nor to defects in MMR proteins, *TP53* mutational status or any histopathological features (Burger *et al*, 2006b).

Other malignancies. Instabilities in tetranucleotide repeats have been reported in both the MSI-H and MSI-L types of gastric tumours, although EMAST has not been specifically investigated in this setting (Kim *et al*, 2001). In a small study of 22 pheochromocytomas (Kupka *et al*, 2008), 2 patients demonstrated MSI in 1 and 2 of three tetranucleotide repeats (D2S443, D16S752 and D21S1436) investigated; both cancers were defined as MSI-L by the Bethesda panel. None of the endocrine tumours in that study exhibited instability in the tetranucleotide markers used, although 13% were classified as MSI-H (Kupka *et al*, 2008).

EMAST was also analysed in one study of non-melanoma skin cancer, where it was found at a high incidence (75%) and was associated with *TP53* or *PTCH* (patched gene) loss of heterozygosity (Danae *et al*, 2002). *PTCH* acts as receptor in the sonic hedgehog signalling pathway, and loss-of-function mutations in *PTCH* contribute to skin cancer development. The *PTCH* gene harbours mutational hot-spot residues and regions, including a slippage-sensitive sequence at the N-terminus (Lindstrom *et al*, 2006).

EMAST frequencies in head and neck cancers have been reported to range from 48% to 56% (Xu *et al*, 2001; Temam *et al*, 2004). Of clinical relevance was one report showing that patients with histologically proven (=R0) radical and curative surgery, but who displayed EMAST at the resection margins, had a higher risk of tumour recurrence (Temam *et al*, 2004). 'Molecular-positive' resection margins may thus be proposed as grounds for more aggressive treatment or surveillance if validated as a broadened concept.

More recently, the length of an AAAG tetranucleotide repeat tract and polymorphisms in a tetranucleotide repeat, both located within the *KCNQ1OT1* gene, have been shown to correlate with an increased risk of breast cancer (Karimi *et al*, 2013) and hepatocellular carcinoma (Wan *et al*, 2013), respectively.

CONCLUSIONS

The molecular mechanisms of EMAST have yet to be clearly unravelled. EMAST induction and its contribution to carcinogenesis may stem from both exposure to external mutagens and malfunctioning intrinsic cellular mechanisms. Experiments have suggested that long microsatellites with higher numbers of repeats may be prone to replication errors at higher frequencies compared with the shorter ones. However, the consequences of EMAST development in

carcinogenesis require further investigation. The results from human solid cancers have led to proposed mechanisms involving DNA repair by *TP53* and *MSH3* and have suggested the immunological involvement of T cells, at least in CRC, although precise mechanistic understanding clearly requires further investigation.

The potential role of EMAST in early detection, prognostication and prediction has been poorly investigated. However, some findings have revealed the potential future use of this marker in cancer. Variations of specific lengths in certain polymorphic tetranucleotide repeats are associated with an increased cancer risk, as recently reported for breast cancer and hepatocellular carcinomas (Karimi *et al*, 2013; Wan *et al*, 2013). The high prevalence of EMAST in the early stages of some cancers, particularly those of the upper respiratory organs and lower urinary tract, may suggest EMAST as a potentially exploitable marker for preventive/early detection screening. In fact, in biopsies from endoscopically normal colons, MSI in mononucleotide and dinucleotide markers has been observed earlier than neoplastic changes (Tug *et al*, 2012). Although markers suggestive of EMAST have yet to be explored in the same manner, they EMAST markers may be found more abundantly, because EMAST occurs at higher frequencies. EMAST may also be generally related to environmental carcinogen exposure and may therefore serve as a marker of exposure or risk in certain cancers.

EMAST in the tissues of apparently tumour-free marginal sites of surgical resections has been correlated with disease recurrence (Temam *et al*, 2004). Therefore, EMAST in the tissues of histomorphologically normal (R0 resected) surgical cancer specimens could be used as a prognostic predictor of disease recurrence in head and neck cancers. Optimal marker selection may also lead to the feasibility of urine analysis for the early detection or postsurgical surveillance of patients with bladder cancer (van Tilborg *et al*, 2012).

Finally, the prognostic value of different forms of MSI in CRC warrants further research (Garcia *et al*, 2012). The 5-year recurrence-free survival trends for EMAST-positive and MSI-L tumours are worse compared with those of MSI-H tumours, suggesting differences in tumour biology that are currently not fully appreciated and that may have consequences for the use of the other available predictive and prognostic markers.

Taken together, these data suggest that EMAST should be further investigated for its potential use in screening and/or as a prognostic and predictive biomarker. In the fields of clinical and translational oncology, important steps need to be taken to improve the understanding of the role of EMAST in cancer. In our view, one unresolved issue remains regarding whether EMAST represents an 'innocent bystander' in cancer or an 'active partner' in carcinogenesis. Based on the emerging findings and cumulative reports discussed above, EMAST may have diverse roles in carcinogenesis and tumour biology at different tumour sites. Nevertheless, common underlying principles may be crucial for understanding cancer behaviour and may yield new tools for prevention, early detection or personalised treatment.

ACKNOWLEDGEMENTS

Work in our laboratory was supported by grants from the Folke Hermansens Cancer Fund, the Mjaaland Research Fund and the Institute for Surgical Sciences, University of Bergen, Norway. We apologise to those colleagues whose work could not be cited due to space limitations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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