



INVESTIGATION OF HLA GENES, HLA MOLECULES AND OTHER FACTORS IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Major histocompatibility complex class I-related chain A (MICA) is a ligand of Natural killer group 2, member D (NKG2D) receptor. Recent studies have shown that MICA is upregulated in tumors from the epithelial origin, playing a key role in immunological surveillance and different alleles are associated with diseases related to NK activity. The aim of our study was to analyse the associations of MICA polymorphism with oral squamous cell carcinoma (OSCC). Twenty seven patients with histologically proven OSCC were included in the study. The majority of patients had G2-G3 tumors according to Anneroth's classification. The control group included healthy subjects from the Bulgarian population. MICA genotyping was performed by PCR-SSO kit (LABType SSO MICA, OneLambda) and PCR-SBT. Our results showed a statistically significant protective association for MICA*12:01 allele ($P < 0.05$, OR=0.07), encoding a full-length protein. Interestingly this allele had a higher frequency in the healthy Bulgarian population compared to other European populations. With the highest frequency in patients with OSCC was observed MICA*08:01 allele, encoding truncated protein. However, the difference with the control group was with a borderline significance ($P = 0.053$). Although our data are preliminary considering the small number of patients analyzed, the associations observed support the model that alleles are encoding truncated, ectopic and soluble MICA molecules play an important role in OSCC by down-regulation of NKG2D on NK and CD8+ T cells leading to aberrant immunological surveillance.

Keywords: biomarkers, oral squamous cell carcinoma, HLA,

INTRODUCTION

The major histocompatibility complex (MHC) region on the short arm of chromosome 6 contains polymorphic genes that play important roles in immune response. MHC encoded molecules were discovered on the surface of white blood cells, so the human MHC was referred to as the human leucocyte antigen (HLA) complex. The HLA genes are grouped into 3 classes. The classical HLA molecules – class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP) include membrane-bound antigen-presenting molecules that act as a ligand for the T cell receptors (TCRs). The presentation of HLA-anchored antigens to T lymphocytes (CD4+ and CD8+) leads to a cascade of immune responses and the activation of the adaptive immunity. HLA class III genes encode a diverse group of molecules with immunological functions such as complement proteins and inflammatory cytokines. Unlike the classical MHC proteins, there is a number of MHC genes that encode molecules that are not involved in antigen presentation and are referred to as non-classical MHC molecules. In 1994, a new set of loci related to MHC class I genes called MHC class I chain-related genes (MIC) were identified independently by Bahram et al. [1] and Leelayuwat et al. [2]. The most polymorphic non-classical class I gene was found to be MICA (major histocompatibility complex class I polypeptide-related sequence A gene). MICA is stress-inducible, highly polymorphic gene that encodes membrane-bound protein acting as a ligand for immune cells including natural killer cells (NK) and specialized cytotoxic T cells ($\gamma\delta$ T and CD8+ $\alpha\beta$ T cells), which express common activating NK cell receptor NKG2D. The transcription of MICA has been detected in all tissue and organs except the brain. However, their restricted protein expression is due to its regulation at the posttranslational

level [3]. In contrast to the ubiquitous expression of classical class I molecules, the MICA protein has a limited tissue distribution. Normally, MICA is constitutively expressed in low levels on gastrointestinal epithelial cells, thymus, endothelial cells, fibroblasts and monocytes [4]. However, MICA expression is induced or up-regulated in stressed conditions such as heat [5], viral/bacterial/infection [6–8], inflammation, DNA damage [9], oncogenic transformation [4, 10] or in autoimmunity [11, 12]. In line with this notion, MICA expression is rarely found on the cell surface of healthy cells lineage. In the absence of cell stress or other pathological conditions, the elimination of NKG2D ligand-expressing cells (NKG2DL) such as MICA is avoided as either NKG2DLs are expressed concomitantly with high levels of inhibitory ligands such as MHC class I molecules or as these cells are inaccessible for NK cells [13]. Thus, membrane expression of MICA ligands marks the aberrant cells and initiates the activation of the immune effector cells that express the NKG2D receptor.

Among the evasion strategies developed by tumors, the metalloprotease dependent shedding of the MICA molecules from tumors (either in exosome form) can hinder the recognition of MICA-expressing transformed cells and also results in systemic down-regulation of NKG2D expression on the cell surface of immune effector cells and therefore leads to evasion of NKG2D-mediated immune response. High levels of the soluble form of MICA (sMICA) have been observed in sera of patients with malignancy and pre-cancerous condition, but in healthy subjects, those molecules are not detected [14–19]. Elevated sMICA levels in cancer patients correlate significantly with the tumor stage and metastasis [20].

Given the important role of MICA in immune activation and surveillance against infection and tumorigenesis, the association between MICA polymorphism and susceptibility to cancer can be used for diagnostic/prognostic and therapeutic purposes. Further studies would provide a better tumour-specific understanding of MICA importance, particularly its evolutionary development, pathobiological roles and mechanisms of action, leading to devise effective therapeutic strategies for diseases mediated by MICA, in particular, for cancer.

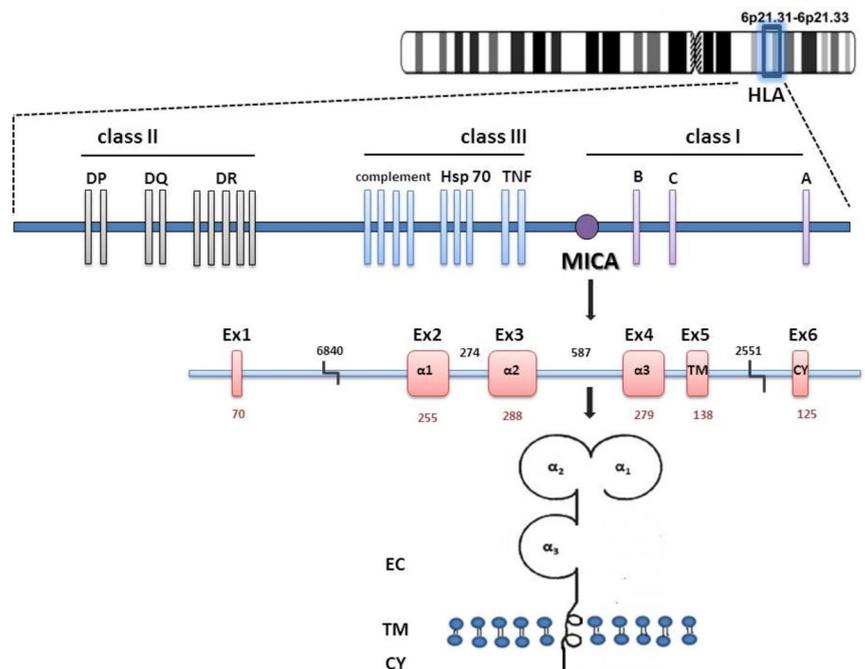
MICA gene, gene expression and polymorphism

The Major Histocompatibility Complex class I-related chain genes (MIC) are a family of proteins encoded within the human HLA region on the short arm of chromosome 6p21.3 [1]. One of the expressed members of this family is the highly polymorphic *MICA* gene (~15.5 kb in size), located between HLA-B and tumor necrosis factor (TNF)-

a(21) (Fig.1). This close proximity to HLA-B gene results in a very strong linkage disequilibrium effect between the two. The intron-exon structure of MICA gene has six exons separated by five introns, of which the first intron is the largest [21]. The domain structure of MICA is similar to those of classical class I molecules, with three extracellular domains, namely $\alpha 1$ (encoded by exon 2), $\alpha 2$ (encoded by exon 3) and $\alpha 3$ (encoded by exon 4), a transmembrane domain (encoded by exon 5) and a hydrophobic cytoplasmic tail (encoded by exon 6) (Fig.1). Unlike the classical MHC class I, MICA do not bind $\beta 2$ -microglobulin or another low molecular weight molecule. The platform formed by the $\alpha 1$ and $\alpha 2$ domains interact directly with NKG2D [22,23]. In spite of substantial sequence diversity, the promiscuous NKG2D binding is enabled by the common site of $\alpha 1\alpha 2$ super domain which consists of structurally conserved patches [24, 25]. NKG2D receptors recognize the human MICA protein through their transmembrane signalling adaptor protein - DAP10, with co-stimulatory functions [26].

Fig. 1. Location of the MICA gene on the short arm of chromosome 6, exon-intron organization of the gene and schematic protein structure of MICA protein.

Intron and exon lengths are in base pairs (bp); The marked introns are not at scale; $\alpha 1$ – $\alpha 3$, external domain; TM, transmembrane domain; CY, cytoplasmic tail.



The MICA gene has a variable expression pattern. MICA protein have limited tissue distribution, and in normal conditions, it is expressed in very low levels in epithelial cells especially in the gastrointestinal tract, endothelial cells and fibroblasts [27]. Because MICA expression is inducible or upregulated in subsequently to harmful events such as cellular stress or viral infection, the molecule have

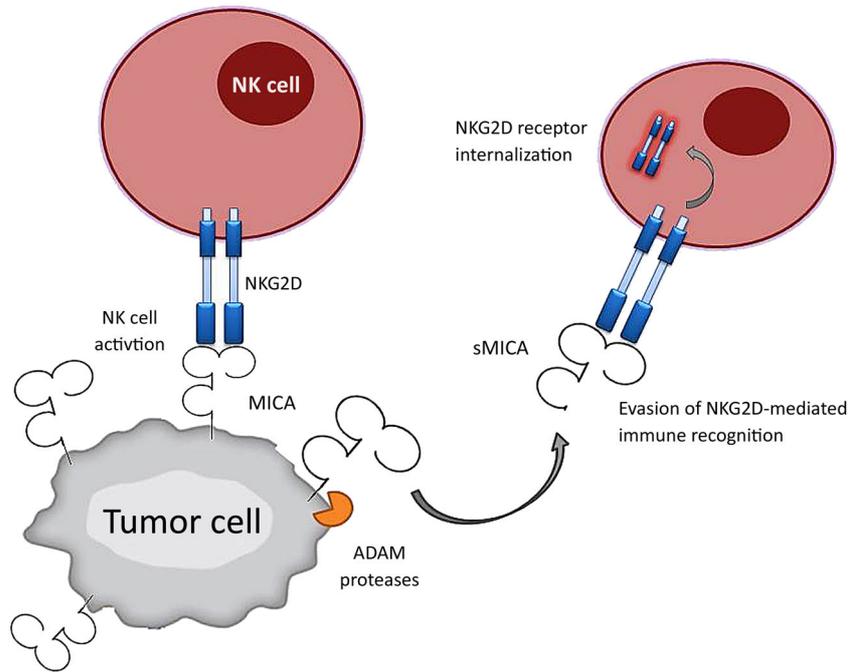
been thought to be stress marker for the cells [5, 7, 9, 28, 29]. Thus MICA through the engagement by NKG2D receptor triggers NK cells, cytotoxic $\gamma\delta$ T cells and antigen-specific CD8+ $\alpha\beta$ T cells, resulting in a range of immune effector functions, such as cytotoxicity and cytokine production [21, 30]. Furthermore, MICA proteins might affect the survival of transplants through activating the complement system [31, 32]. This protein is broadly expressed on epithelial tumors and on some hematopoietic malignancies [33–35], and this has raised the possibility of the role of MICA as tumor-associated antigen [36]. The recognition of the MICA ligands on tumor cells by the NKG2D receptor induces the cytotoxic activity of the immune effector cells (34) and the subsequent lysis of their tumor targets. Besides being regulated by cell-intrinsic mechanisms, the expression of MICA is greatly influenced by factors, released in the tumor microenvironment that favoring tumor growth such as interferons [37, 38]. However, taking together with the variations in the expression levels of MICA in different physiological and pathophysiological contexts warrants that there is no coherent pattern of MICA expression in several human tumors compared with the respective normal tissues [39].

In established MICA-expressing human tumors, the NKG2D mediated immune surveillance is thought to be antagonized by the secretion of soluble MICA molecule (sMICA). This is a mechanism, called “MICA shedding”, for tumor cell immune escape through the saturation of NKG2D receptors on cytotoxic cells [10]. On the surface of tumor cells the proteolytic cleavage of MICA has shown to require the recruitment of disulphide isomerase/chaperon, endoplasmic reticulum protein 5 (ERp5), through which is induced a conformational change enabling the proteolytic cleavage of MICA by ADAM proteases (a disintegrin and metalloproteinase) [40, 41] (Fig. 2). This shedding has been shown to be promoted by the palmitoylation of key cysteine residues in MICA cytoplasmic tail, thus the ligand is protected from degradation, and this process is also an important determinant for it being sorted to the cholesterol/caveolin-1-enriched microdomain [42]. The cleaved MICA then interacts with NKG2D, which, in turn, induces the internalization and degradation of the receptor-ligand complex and thus suppresses NKG2D-mediated host innate immunity [10, 43].

Fig. 2. Mechanism of MICA shedding.

NK, natural killer cells; sMICA, soluble MICA molecule;

Adapted from „MICA polymorphism: biology and importance in immunity and disease“ [10]



Besides MICA shedding, microRNAs (miRNAs) in the tumors (miR 17 5; miR 20a; miR 93) might downregulate MICA to avoid immune recognition [26], and therefore the downregulation of those specific miRNA can contribute to the increased expression of MICA at the surface [44]. Further, in certain types of cancers, MICA is sorted and shed into exosomal compartment due to a polymorphic variant (MICA*008 - A5.1 microsatellite polymorphism) which makes them very potent for down-modulating the NKG2D receptor in effector immune cells and contributing to a diminished NK cell mediated antitumor response [45]. The mechanism of shedding and release of sMICA extracellularly (free or the exosome form) by tumors can be detected in high levels in sera of patients with various malignancies, and there is evidence that the levels of sMICA correlate significantly with cancer stage and metastasis [20]. In addition, determination of soluble MICA levels may be implemented as an important immunological diagnostic and prognostic marker in patients with epithelial malignancies [46].

The complexity of MICA is further increased by his polymorphic nature. MICA is the most polymorphic non-classical class I gene known with 105 alleles having already been reported (according to international ImMunoGeneTics information system (IMGT)/HLA Database) as defined by the combination of the sequence polymorphism of the coding region of the mature protein and new alleles being continuously identified. In contrast to the polymorphism in HLA class I molecules that are located predominantly in the prox-

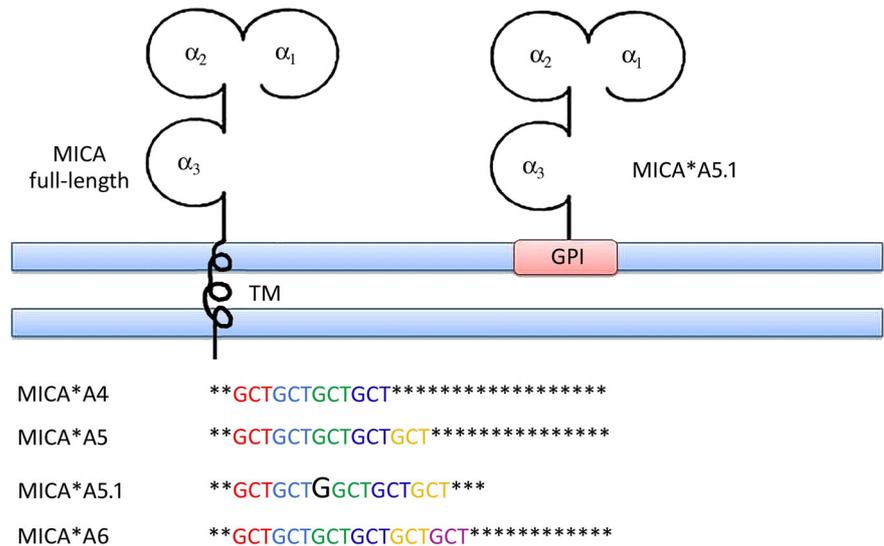
imity of antigen binding groove, the MICA polymorphism is dispersed to all the three extracellular domains (with the greatest variability in the α_2 domain, encoded by exon 3) and variations of polymorphism is observed in the TM region for several MICA alleles despite having identical extracellular domains [4, 10, 47]. Moreover, unlike the polymorphic positions of HLA that typically consists of several amino acids, MICA polymorphism is generated mainly by single amino acid substitutions (except positions 90 and 91) giving rise to dimorphic positions (except residues 156 and 251). The polymorphism in MICA gene has been shown to affect the affinity for NKG2D [23]. Accordingly, a non-synonymous Methionine to Valine change (SNP rs1051792 A>G) at position 129 of the α_2 domain categorizes MICA alleles into “MICA-129 met”, which is a strong binder of NKG2D receptor (10- to 50-fold greater capacity to complex NKG2D) and “MICA-129 val” having weak binding ability [48, 49]. Furthermore, MICA-129 met isoform is less efficiently expressed on the cell surface as compared to the MICA-129 val variant.

On the other hand, the transmembrane domain (TM) of MICR encoded by exon 5 harbors a variable number of short tandem repeat (GCT) leading to polymorphism consisting of four, five, six or nine alanine (Ala) residues or five repetitions of GCT with an additional guanine insertion (after two GCT triplets), designated as A4, A5, A6, A9 and A5.1, respectively [50]. The extra guanine insertion in A5.1 allele (most commonly found in MICA*008 allele, but also in MICA*023, MICA*028 and MICA*054) causes a frameshift mutation resulting in a premature stop codon that, in turn, truncates 10 amino acids of the TM domain as well as the hydrophobic cytoplasmic tail (50), leading to the expression of a truncated protein. These alleles have different biological properties, compared with full-length alleles, e.g. recruitment into exosomes, which makes them very potent for down-modulating the NKG2D receptor in effector immune cells [45]. The distinct biological features of the truncated MICA*008 are due to that the protein is attached to the plasma membrane via GPI (glycosyl phosphatidyl inositol) anchor [45] (Fig. 3). The cytoplasmic tail-deleted MICA-A5.1 gene product is aberrantly transported to the apical surface of human intestinal epithelial cells instead of the basolateral surface where the interaction with intraepithelial T and NK lymphocytes takes place (51). Thus, MICA-A5.1 carriers may have an aberrant immunological response. Incubation of NK cells with MICA-A5.1 (MICA*008) containing supernatant triggers significantly more NKG2D downregulation than the supernatant containing full-length sMICA molecules [45]. These findings highlight the significance of prognosis of the presence of MICA in diseases such as cancer and the need of considering

whether the molecule being analyzed is a TM or a GPI-anchored and therefore release in exosomes or soluble.

Fig. 3. Schematic structure of full-length MICA molecules with TM domain and truncated MICA*A5.1 molecule attaches to the plasma membrane via a GPI anchor and has no cytoplasmic tail.

Short tandem repeats (STR) of the TM region are shown below.



MICA association with Oral Squamous Cell Carcinoma (OSCC)

Oral cancer refers to cancer occurring between the vermilion border of the lips and the junction of the hard and soft palates or the posterior one-third of the tongue. The sixth most common malignancy worldwide and the most common oral cancer (encompasses at least 90% of all oral malignancies) is squamous cell carcinoma (OSCC), also known as epidermoid carcinoma [52]. This type of cancer is a solid tumor originating from squamous cells that form the surface of the skin lining of hollow organs in the body and line the respiratory and digestive tracts.

Like other epithelial malignancies, OSCC is a heterogeneous group of tumors that arise from the accumulation of a series of genetic and epigenetic alterations, that affects cell cycle and proliferation. A major step in oncogenesis is the ability of tumor cells to evasion of immune response as well as the production of immunosuppressive cytokines, and for this reason, tumor immunity attaches considerable attention in the research area. Being one of the most important and most polymorphic genetic components of the body's immune function, both HLA class I and class II genotypes have been associated with tumor susceptibility and implicated in the development of squamous cell carcinoma [53]. In addition to class I and class II HLA genes, the closely related to HLA-B gene, MICA are also involved in the immune system regulation, and associations of MICA polymorphism have been observed for several diseases, including tumor transformation. MICA is expressed under abnormal conditions such as viral infection or

cell transformation. Therefore, several carcinomas of epithelial cell origin express MICA on their cell surface and in some tumor types of soluble MICA has been detected in the sera of patients [5, 33, 54–57]. Dependent on whether MICA is expressed at the cell surface or as a soluble molecule, upon interaction with the NKG2D receptor presented on immune effector cells, different immunological responses might occur. Tumor cell with surface expression can activate the immune system while soluble MICA might oppose the immune system through blocking of the NKG2D receptor [29, 57, 58].

Certain MICA–STR alleles have been associated with immune-mediated diseases [59–63] and different type of cancers (table 1). The association between the MICA STR polymorphism and risk of OSCC has been investigated in three populations (Taiwanese, Dutch, Japanese), with conflicting results and no firm conclusions can be drawn for the role of MICA polymorphism in the susceptibility for OSCC. A case-control study in Taiwanese population found that carriers of MICA-A6 allele may have a higher risk for development of OSCC [OR=2.64(1.39-5.02), $P_c=0.01$] [64]. In another study of the Dutch population MICA-A9 allele was significantly associated with decreased risk of development of OSCC [$P_c=0.01$] [54]. On the other hand, in two studies of the Japanese population was reported the positive association of MICA-A5.1 allele with the development of oral cancer [OR=1.66(0.82-3.42), $P=0.021$; OR=1.37(0.61-3.62), $P=0.038$] [19, 65], furthermore homozygous patients were found to have higher levels of sMICA and lower survival rate.

The conflicting results can be due to that most of the investigations were performed with small sample size, single-ethnic populations and are focused mainly on certain polymorphism in the TM domain (exon 5) of the MICA molecule. Furthermore, the heterogeneity in the results could also come from variations in study design, differences in biological effect between populations due to modification of environmental factors, lack of adjustment for known risk factors and issues related to multiple significance testing [4]. In order to further our understanding of the role of MICA polymorphism in different types of cancers, it is imperative to require larger sample size, a more careful study design, data analysis and more detailed analyzes of the polymorphisms in exons 2, 3 and 4 which are coding the extracellular part of the MICA molecule, containing the motives which recognize the NKG2D receptor. OSCC is mainly treated by operation supplemented by chemo-therapy method, however, the facial morphology of patients could be severely affected which leading to the disorder of chewing, swallowing, breathing, language, etc. [66]. Given the important role of MICA in immune activation and surveillance against infection and tumorigenesis, the detailed study of the association between MICA polymorphism and susceptibility to cancer and the understanding of MICA pathogenesis and mechanisms of action, should lead us to devise effective therapeutic strategies for cancer mediated by MICA and therefore manipulations like operations or chemotherapy can be reduced or avoided.

Table 1. Association of MICA polymorphism and different type of cancers (summary of studies)

Type of cancer	Population	Number		Exons 2-4	TM domain	Allele association	OR	P	P _c	Ref
		Case	Control							
Breast cancer	Iranian	110	110		-	MICA*A4	0,46	0.04	NA	(67)
					-	MICA*A5.1	0,43	0.03		
					+	MICA*A6	1,87	0.03		
Naso-pharyngeal carcinoma	Chinese	218	196		-	MICA*A5.1	0,59	1.0×10^{-3}	6.0×10^{-3}	(68)
				+	MICA*A9	2,53	1.7×10^{-5}	1.0×10^{-4}		
	Tunisian	130	180	-		129Met/Val	0,53	NA	0.02	(69)
			+		129Val/Val	1,87	NA	0.02		
Colorectal cancer	German	79	306		+	MICA*A4	NA	0.015	NA	(70)
Leukemia	Chinese	107	162		-	MICA*001 (*A4)	NA	0.01	NS	(71)
					+	MICA*027 (*A5)	NA	1.0×10^{-3}	<0.01	
					-	MICA*008 (*A5.1)	0,59	4.7×10^{-3}	0.03	
Gastric cancer	Taiwanese	107	351		+	MICA-A9	NA	4.0×10^{-3}	0.02	(72)
Ulcerative colitis	Spanish	340	636		-	MICA*A4 HLA-B*52/MICA*A6	0.643	0.003	NA	(73)
				+			2.914	0.001	NA	
OSCC	Japanese	83	132		+	MICA*A6	2.69	NA	1×10^{-5}	(74)
	Taiwanese	67	351		+	MICA*A6	2.64	0.002	0.01	(64)
	Dutch	56	106		-	MICA*A9	NA	NA	0.01	(54)
	Japanese	123	188		+	MICA*A5.1	1.66	0.021	NA	(65)
	Japanese	80	70		+	MICA*A5.1	1.37	0.038	NA	(19)

OR, odds ratio; Pc, P value after correction for multiple testing; NA, not available; +, positive allele association with the development of cancer; -, protective allele association (negative association) against the development of cancer. Adapted from „MICA polymorphism: biology and importance in cancer“ [4]

CONCLUSION

In the last years subject of increased research interest are the pathogenetic mechanism underlying the development of lesions and especially tumors. Immunosurveillance is an important part of the immune response not only against pathogens but also against cancer. As one mechanism, the cytotoxic lymphocytes NK and CD8+ T and $\gamma\delta$ T cells with their common NKG2D receptor can detect cell surface molecules whose expression is induced in infected or transformed cells. The recognition of such „stressed“ cells is enabled by binding of NKG2D to MHC class I chain-related gene A (MICA), which is upregulated or expressed de novo in the course of viral infection or malignant transformation. Thus MICA/NKG2D interaction is an effective mechanism for protecting the organism from tumorigenesis. MICA gene encodes membrane-bound protein which is involved in both innate and adaptive immune response through activating the cytotoxic activity of NK cells and CD8+ T cells. MICA molecules are highly polymorphic since its alleles vary among individuals and may confer variable disease susceptibility, analyses of MICA alleles may be useful in cancer investigation.

Tumor cells have evolved a mechanism to avoid the immune response mediated by MICA/NKG2D interaction –they released a soluble form of MICA (sMICA) through proteolytic cleavage of the extracellular part of the molecule. The shedding of sMICA by tumors hinders recognition of the MICA-expressing tumor cells and results in systemic downregulation of NKG2D and evasion of NKG2D-mediated immune recognition [57].

The associations of MICA polymorphism with OSCC

have been studied in only 3 populations, and the obtained data are controversial. Since these studies include a small number of individuals, they are focused on certain short tandem repeats (STR), and the obtained data are very contradictory, there is no conclusive conclusion about the role of MICA polymorphism in the development of oral squamous cell carcinoma. Once there is evidence that a particular variant in a given gene is associated with risk of certain cancer, the probability that other functional variants in the same gene also modify cancer risk is markedly enhanced [75]. Therefore, further studies in the MICA gene and flanking region will be required to allow for the discovery of rare causal variants in MICA affecting the risk of OSCC and to clarify the role of MICA allelic polymorphism as a predisposing factor for the development of cancer.

The obtained data from the past years and the witnessed substantial progress in our knowledge of MICA alleles association with cancers should be directed to the discovery of drugs and the development of therapeutic strategies. The potential therapeutic targets for MICA shedding seem to be ERp5, and soluble MICA [4] and the use of antibodies against MICA could reduce levels of soluble MICA [76], that may bolster the NKG2D/MICA system against malignancies.

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