Augmented serum levels of soluble MICA and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions

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Abstract

Background
Cervical cancer represents the second cause of cancer-related death worldwide. NK and cytotoxic T cells play an important role in the elimination of virus-infected and tumor cells through NKG2D activating receptor, which can promote the lysis of their target cells by binding to cell surface MICA. Increased serum levels of MICA have been found in patients with epithelial tumors. This study was aimed to compare values of soluble MICA in serum and NKG2D expression on NK and T cells from patients with cervical cancer or precursor lesions with those from healthy donors. Patients with either cervical cancer or precursor lesions as well as healthy donors were included in the study. Serum and peripheral blood were collected before standard treatment. sMICA was measured by ELISA and NKG2D expression by flow cytometry.

Results
Significant amounts of sMICA were detected in sera from nearly all patients. We also found a decrease of NKG2D expression in cervical lesion groups when compared to healthy donors. Correlation analysis demonstrated on one hand, a positive relationship between high sMICA levels with the progression of the lesion. On the other hand, high sMICA was negatively correlated with reduction of NKG2D-expressing T cells.
Conclusion

Our results show important high levels of sMICA in almost all patients. Conversely, we observed a noticeable diminution of NKG2D expression either NK or T cells from all cancer patients. Thus, our results suggest that sMICA might represent an additional mechanism responsible for the immune evasion in cervical cancer patients.
Background

Cervical cancer is the second most common malignant tumor in women worldwide, and the first tumor in developing countries including Mexico (1-3). Infection with high-risk human papilloma virus (HPV) is considered the major etiological factor of HPV-related premalignant lesions and cervical cancer (4-7). Virtually all cervical carcinoma patients (99.7%) have shown to be HPV-DNA carriers (8). Although the HPV prevalence is very common in sexually active women (9), the infection in the majority of cases is transient, clearing in a short period of time without progression to clinical lesions (10-12). In a minority of cases, HPV presence is established as a persistent infection. Precisely, it is thought that viral persistence leads to progression from low-grade squamous intraepithelial lesion (LSIL) to high-grade squamous intraepithelial lesion (HSIL) and eventually to invasive carcinoma (13-16). The progression of the lesions may involve an adverse tumor environment, wherein the mucosal immune response may be unable to eradicate malignant cells.

The innate immune response is considered to be the first line of defense at mucosal surfaces. NK cells are an important arm of the innate immune system specialized for killing virus-infected and tumor cells (17). The activity of NK cells is tightly regulated by a complex balance of inhibitory and activating receptors (18). NKG2D is a C-type lectin-like activating receptor encoded within the NK gene complex on human chromosome 12 (19). NKG2D is expressed in almost all NK cells and a variety of T cell subsets such as CD8+ T cells(20). NKG2D can
promote tumor lysis by binding to a recently identified family of cell surface ligands encoded by the MHC class I chain-related (MIC) genes (21-23). MIC proteins are a novel family of non-classical MHC class I molecules. The MIC family includes MICA, which is a highly polymorphic functional cell-surface protein (24). Similar to classical HLA class I molecules, MICA also contains three extracellular domains (α1-α3); however, MICA neither associates with β2-microglobulin nor binds antigen peptides. Under physiological conditions, MICA expression is almost restricted to the gastrointestinal epithelium (25, 26); nevertheless, MICA is overexpressed in several epithelial tumors (27-30). This finding has led to propose that MICA is a stress cell marker in epithelial nascent tumors (26).

Available evidences suggest that NKG2D engagement by MICA induces proliferation, survival, and, cytotoxic activity in NK cells (31, 32). Consequently, NKG2D/MICA interaction may represent an important activation pathway to trigger the immune attack against tumor cells (30, 33). However, it has been demonstrated the shedding of MICA from tumor cell surface in a variety of malignant epithelial tumors, including advanced hepatocellular carcinoma, colon, prostate, renal, breast cancer, as well as hematopoietic tumors (34-38). Such phenomenon provokes accumulation of soluble MICA (sMICA) in serum leading to NKG2D down-modulation by facilitating its internalization and lysosomal degradation. This has been proposed to be a novel evasion mechanism of cancer cells to the NK-mediated tumor surveillance (39).
In this study, we formulated the hypothesis that soluble MICA (sMICA) level increases concomitantly with the natural history of cervical cancer, which progresses from intraepithelial precursor lesions toward invasive cancer. To address this assertion, we compared the sMICA levels in blood samples from patients with cervical cancer and precursor lesions with those from healthy donors. We further explored the number of peripheral NK and T cells expressing the NKG2D receptor on cell surface. Finally, we used a Spearman or Pearson analysis to correlate the sMICA levels with the stage of the lesion or with NKG2D expression.
Results

Ten patients with established histopathological diagnosis of uterine cervix invasive squamous cell carcinoma were enrolled at this study. The group of squamous intraepithelial lesions was integrated by 17 patients diagnosed as high-grade (7 individuals) or low-grade (10 individuals). We also included a control group matched in age/gender as shown in Table 1. Clinical and laboratory parameters confirmed that patients and controls did not have any autoimmune or blood disorder that could alter our study variables (sMICA level and NKG2D expression).

*Serum value of soluble MICA is preferentially augmented in patients with either cervical cancer or precursor lesions.* We investigated sMICA concentrations in sera from cervical carcinoma patients and SIL (including both high-grade and low-grade lesions). Additionally, we also tested sMICA in healthy individuals. In order to normalize the sMICA values, we changed the data (expressed as pg/mL) to log$_{10}$. As shown in Figure 1, we found a significantly higher sMICA concentration in both precursor lesion and cervical cancer patients when compared to healthy donors (Mann-Whitney test, $p<0.005$ in LSIL, $p<0.030$ in HSIL, and $p<0.001$ in cancer group). Interestingly, whereas more than 50% of healthy individuals showed to have trace sMICA levels (lower than 1 pg/mL), cancer patients did not show to have comparable values to those individuals; even more, the highest level of sMICA was found in this group (4.56 pg/mL).
We also estimated a potential relationship between sMICA levels and the stage of the lesion. Sperman correlation showed that sMICA concentrations were significantly positively correlated with the severity of the lesion (Rho=0.519; p<0.001). These results point out that sMICA is preferentially detected early in the development of cervical lesions and this molecule appears to be augmenting in line with the progression toward cancer.

*Reduced numbers of NKG2D expressing-NK and T cells in patients with either cervical cancer or precursor lesions.* NKG2D plays an important role in the immune recognition of tumor targets after engagement by MICA molecules (31, 32). Nevertheless, there are evidences showing that circulating sMICA interferes with NKG2D expression on NK and T cells (39). Owing to the important increase in the sMICA level detected in sera from patients with cervical cancer and those with precursor lesions, we investigated the expression of NKG2D receptor in both peripheral NK and T cells. First, we evaluated the percentage of either CD56⁺ NK or CD3⁺ T cells in all patients and healthy donors. The results fell into the normal range in all groups (data not shown). In order to determine NKG2D expression in NK cells, we gated the CD56⁺ cell population. As shown in Figure 2A, we found an NKG2D-positive cell percentage of 50.4 in control group, 28.6 in LSIL, 40.9 in HSIL and 27.5 in cancer group. Statistical comparison between cancer and control groups revealed a significant difference (p<0.03).
We also analyzed the CD3\(^+\) region to determine NKG2D expression in T cells. We observed a decrease in NKG2D expression according with the severity of the lesion as it can be observed in Figure 2B, which shows a 24.9% of NKG2D-positive cells in the control group. In contrast, LSIL, HSIL, and cancer groups showed a percentage of 15.7, 12.2, and 8.2, respectively. Statistical analysis showed a significant difference in both precursor lesion and cancer groups when compared to healthy individuals (p<0.04, p<0.01, and p<0.005 to LSIL, HSIL, and cancer groups, respectively).

*Circulating sMICA can reduce the number of NKG2D expressing-cells.* It has been well established that sMICA in serum from patients with cancer induces down-modulation of NKG2D surface expression (39). For that reason, we additionally investigated a potential correlation between increased soluble MICA levels in serum and the decrease of NKG2D expressing-cells. Importantly, sMICA levels were negatively correlated with number of NKG2D expressing-T cells (r=−0.359; p=0.015) as shown in Figure 3. In contrast, we did not observe any significant correlation between sMICA concentration and NKG2D expressing-NK cells, albeit, r value was negative (data not shown). Most importantly, more than a half of the patients with cervical cancer showed a substantial decrease of NKG2D-expressing NK cell number as shown in Figure 4.

Finally, we used a multivariate regression analysis to determine if high sMICA concentrations and diminished NKG2D expression could act in concert to predict
the progression of the lesions advancing toward cancer. The results derived from that analysis (Table 2) show that both categories (high sMICA level and NKG2D down-modulation) were significantly strongly related with the progression of the lesion ($R^2=0.467; \ p<0.001$). The importance of these results, is that $R^2$ predicts that the variation in the progression of the lesions advancing toward cancer, can be explained in a 46.7% as a result of an increase in the sMICA concentrations, and a decrease of NKG2D-expressing T cell number (as we did not find any significant correlation when we applied the analysis to NKG2D-expressing T cells).
Discussion

Recent reports have revealed a new tumor evasion strategy through MICA releasing from malignant cell surface in different human tumors (34-37). In this study we also found significant sMICA levels in patients with cervical cancer. Proteolytic shedding has been proposed as the key mechanism by which MICA is released from cell surface, similar to the cleavage occurring for other membrane-bound proteins. By using different protease inhibitors, it was elucidated that metalloproteinases were responsible to cleave the MICA $\alpha_1\alpha_2\alpha_3$ extracellular domain from cell surface in several tumor lines (44). This finding may have implications in cervical cancer owing to previous data in our laboratory have shown an extensive proteolytic activity in cervical tissue extracts from patients with cervical cancer and precursor lesions; even more, the activity was increasing from precursor lesions toward malignancy (45). We also particularly found that metalloproteinases were the predominant type responsible for the proteolysis.

The above information, in conjunction with our current sMICA data suggest that metalloproteinases besides to play an important role in invasion, metastasis, and angiogenesis processes, may also have an additional function in cervical cancer progression by facilitating the escape of MICA shedding-tumor cells from the immune attack. It is particularly important to identify the metalloproteinase responsible of shedding MICA from cell surface in cervical cancer as well as explore whether that proteolysis-mediated shedding additionally enlarges the immune suppression carried out by different proteases as we have also
demonstrated in a previous report (46). Importantly, in that study we demonstrated an imbalance of mitogen-induced lymphocyte proliferation following to the administration of cervical carcinoma extracts or purified enzymes including IV-type collagenase. Thus, it is feasible to rationalize that different evasion mechanisms may contribute to the immunosuppression observed in cervical cancer patients.

On the other hand, we observed a higher amount of sMICA in patients with low-grade intraepithelial lesion in comparison with healthy individuals; this amount was over-increasing according with cancer progression. This finding is in agreement with recent results obtained by Wu et al., whom detected elevated amount of sMICA in patients with prostate cancer (36). Moreover, a significant higher level was seen in patients with more advanced disease, suggesting that the shedding of MIC molecules may contribute to prostate cancer progression. Thus, consistent with Wu et al., we can also speculate that high concentration of circulating sMICA may represent one of the mechanisms responsible for immune evasion observed in patients with cervical cancer.

Different studies have proven down-modulation of HLA class I expression during cervical cancer progression (47, 48). Hence, it is feasible to assume that NK cells may represent an important immune arm against cervical cancer. Different activating receptors confer to NK cells the capacity to kill virus-infected or tumor cells (49, 50); one of these receptors is represented by NKG2D, which can recognize different ligands including MICA molecules; thus, NKG2D could have a significant role in anti-tumor immune response (21-23).
Recently, Doubrovina et al., have established that sMICA in serum from patients with colon adenocarcinoma down-regulate NKG2D expression on NK cells via its internalization and subsequent lysosomal degradation (35). We also observed a reduction of NKG2D-expressing NK cells in patients with cervical precursor lesions and invasive cancer. Despite the fact that we did not reach a significant statistical correlation, it is important to point out that a cancer patient with considerably high sMICA level (as high as 30 ng/mL) did not show any NKG2D expression on NK cells (as it is shown in Fig. 4).

Additionally, other factors could also down-regulate surface NKG2D expression. Lee et al., have provided evidences showing that TGF-β1 present in plasma of lung and colorectal cancer patients impairs NK cells activity via NKG2D down-modulation (51); this cytokine is largely produced by many tumor cells and is also common in cervical squamous intraepithelial lesions (52). It has been shown that HPV-11 transformed human tissue over expresses TGF-β1 (53) and particularly benign cervical lesions have been associated with HPV-6 or -11. Thus, these data could explain why LSIL group showed a lower number of NKG2D-expressing NK cells than observed in HSIL patients. Therefore, sMICA and TGF-β1 could collectively synergize to down-modulate surface NKG2D expression.

It has been recently observed that NKp44, another important receptor confined only to NK cells upon activation, becomes down-regulated on NK cells from healthy
donors or patients with cancer upon exposition to sMICA-containing serum (35). Taken into account that NKG2D and NKp44 on cell surface could be down-regulated by sMICA, it is viable to consider that circulating sMICA could be affecting the integration of different crucial triggering signals in NK cells. Collectively, these alterations may be contributing to the incomplete NK cells-mediated cytolytic function in patients with cancer.

NKG2D expression is not only confined to NK cells, but it is also expressed on CD8+ T cells, wherein this receptor confers co-stimulatory signals [31]; for this reason, we also evaluated surface NKG2D expression on T cells. As it was expected, NKG2D-expressing T cell number was lower than observed in NK cells; the reason for that difference may be explained owing to a limited marginal number of cytotoxic T cells is expressing NKG2D. In parallel with the observed in NK cells, we also found a reduction of NKG2D-expressing T cells in patients with either precursor lesions or cancer; even more, these results showed to be significantly correlated with the increase of sMICA level. As a result, it is feasible to consider that CD8+ T cells with severe defects in co-stimulatory pathways (for instance defects conferred through NKG2D-mediated signaling) may not be fully activated; in consequence, defective T cells may promote viral or malignant disease progression such as HPV-associated cervical lesions.

Finally, the results derived from the multivariate regression analysis were able to accurately predict that high sMICA levels and low NKG2D-expressing T cell number act in concert during the progression of the lesions advancing to cancer.
If confirmed in a greater number of cervical lesion patients, both high sMICA level and NKG2D down-modulation could represent a new list of predictive factors involved in the different stages during cervical cancer progression.
Conclusion

Here we provide evidences that sMICA may represent a novel immune evasion mechanism through natural history of cervical cancer. Therefore, targeting of the specific proteases involved in MICA shedding from cell surface or directly blocking sMICA in circulation could be a clinical important strategy to enhance the NK and T cell-mediated activity against cervical cancer.
Methods

Patients

According with the FIGO (International Federation of Gynecology and Obstetrics) System of Clinical Staging (40, 41), we included 10 patients with invasive squamous cell carcinoma of the uterine cervix and 17 patients with squamous intraepithelial lesions, which were classified as high-grade squamous intraepithelial lesion (HSIL) or low-grade squamous intraepithelial lesion (LSIL) following the 2001 Bethesda System for reporting cervical or vaginal cytologic diagnoses (42, 43). All patients enrolled in the study were first subjected to colposcopic evaluation. Ten age/gender matched healthy donors were also included as control group. All patients were attended at the Oncology and Gynecology Department, Hospital Civil de Guadalajara, Mexico. The study was approved by the Biomedicine Science Committee, in accordance with the guidelines of the World Medical Association Declaration of Helsinki (adopted by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000).

Biological specimens including blood samples were taken from all patients and healthy donors prior written informed consent. Peripheral blood (PB) was collected in heparinized tubes to separate peripheral blood mononuclear cells (PBMC). PB was also obtained without heparin in order to separate serum, which was stored at −20 °C until use.
Quantification of sMICA in serum

MICA ELISA kit (Immatics Biotechnologies, Tübingen, Germany) was used to detect sMICA in serum samples, according with the manufacturer protocol. This method is based in two anti-MICA mAbs binding to different MICA domains. Maxisorp 96 well flat-bottom plate was coated with AMO-1 capture anti-MICA mAb at 5 μg/mL in phosphate buffer saline (PBS) at 4 °C overnight and blocked by addition of PBS-BSA (15%) for 1 h at 37 °C. After washing with PBS-Tween (0.05%), standard serial dilutions (recombinant MICA*04 ranging from 10 to 20000 pg/mL) and serum samples were added to each well followed by plate incubation for 2 h at 37 °C. Previously, patient sera were diluted at 1:3 in PBS-BSA (7.5%). After that, BAMO-3 detection mAb at 1 μg/mL in PBS-BSA (7.5%) was added and incubation was continued for 2 h. HRP-conjugated anti-mouse IgG2a Ab (Southern Biotechnologies, Birmingham, AL, USA) diluted at 1:10000 in PBS-BSA (3.75%) was added and incubation was followed for 1 h at 37 °C. Color was developed using tetramethylbenzidine system (KPL, Gaithersburg, MD, USA) at room temperature for 25 min. The reaction was stopped with phosphoric acid (1M) and the $A_{450}$ was determined. Absorbance values by duplicate were plotted against dilutions and expressed as pg/mL.

NKG2D expression on NK and T cells

NKG2D surface expression was evaluated by flow cytometry. Briefly, PBMC were obtained by using Ficoll-Hypaque density gradient centrifugation. After isolation, PBMC were adjusted at $8 \times 10^5$ cells/mL and incubated with mouse anti-NKG2D
primary mAb (kindly donated by Professor Alessandro Moretta, University of Genova, Italy) for 30 min at 4 °C. Cells were washed with PBS and incubated with goat anti-mouse IgG FITC-conjugate as secondary reagent for 30 min at 4 °C in darkness. Afterward, cells were washed and incubated with PE-conjugated anti-CD56, and PC5-conjugated anti-CD3 mAbs; matching isotype controls were also included. Finally, cells were fixed with 0.5 formaldehyde solution. A three-color approach in an EPICS XL-MCL flow cytometer (Beckman Coulter, Krefeld, Germany) was used with CD56 and CD3 gating to determine the NKG2D expression percentage.

Statistical analysis

Data to report sMICA levels and percentage of NKG2D-expressing NK and T cells were expressed as mean±SEM. Statistical comparison among different groups was performed by using non-parametric test (Mann-Whitney test). Spearman or Pearson analysis was performed to correlate sMICA levels with the stage of the lesion or with NKG2D expression, respectively. Additionally, a multivariate regression analysis was achieved to evaluate if sMICA and NKG2D expression act in concert during the cervical cancer progression. A 95% confidence interval (p<0.05) was considered statistically significant.
Authors’ contributions

NAG performed the whole of the experiments described in the project, searched scientific literature, contributed with the draft and edited the manuscript; ADN contributed to the planning of the project; participated in its coordination and provided valuable scientific suggestions; ATA was the core in the flow cytometry experiments and performed research; ACA participated in the design of the study and contributed to the review of the manuscript; OGR contributed to the draft of the manuscript and helped to the edition; LJS, and AAL oversaw the experiments and contributed with scientific ideas; RTS supported with the statistical analysis of all the results; VDR, and TGI helped in ELISA experiments; ABC and GHF contributed with scientific ideas and research; STA conceived and designed the theoretical framework of the study, provided scientific guidance throughout the project and wrote the manuscript. All authors read and approved the final manuscript.

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References


Figure 1. Serum sMICA is elevated in cervical cancer patients. sMICA in control (n=10), LSIL (n=10), HSIL (n=7), and cancer (n=10) groups was determined by using an ELISA kit and expressed as the mean of two measurements per sample. Short horizontal lines indicate the mean value (ng/mL) in each group (0.65 in control group, 4.37 in LSIL, 7.04 in HSIL, and 7.37 in cancer group). Statistical analysis among all groups was performed by Mann-Whitney test. Significant differences were found in both lesions and cancer groups when compared to healthy donors ($p \leq 0.005$ in LSIL, 0.030 in HSIL, and 0.001 in cancer group).
Figure 2. NKG2D-expressing NK and T cells. PBMCs were incubated with anti-CD3, anti-CD56 and anti-NKG2D antibodies. Flow cytometry three-color analysis was carried out to determine the percentage of NKG2D-positive cells. **A)** NKG2D-expressing NK cells. Mean values: 50.4 in control group, 28.6 in LSIL, 40.9 in HSIL and 27.5 in cancer group. Mann-Whitney test revealed significant differences among healthy individuals when compared to LSIL, and cancer groups (p<0.01, and p<0.03, respectively). **B)** NKG2D-expressing T cells. Mean values: 24.9 in control group, 15.7 in LSIL, 12.2 in HSIL and 8.2 in cancer group. Significant differences were found among healthy individuals when compared to LSIL, HSIL, and cancer groups (p<0.04, p<0.01, and p<0.005, respectively). The box plots represent each study group. Medians are represented as thick horizontal lines, 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. *Extreme values.
Figure 3. Establishment of a relationship between high sMICA levels and NKG2D down-modulation. Pearson analysis established that sMICA levels were significantly negatively correlated with number of NKG2D-expressing T cells (10 healthy donors, 17 precursor lesion patients and 10 cervical cancer patients were included).
Figure 4. Representative histograms of NKG2D expression on NK and T cells. The number of NKG2D-expressing cells appears to be dramatically diminished in accordance with the cervical cancer progression. The same tendency is observed either NK or T cells (full line: isotype control Ab, empty line: anti-NKG2D Ab).
Figure 1

The graph shows the Log$_{10}$ sMICA (pg/mL) concentrations for different subjects: Healthy, LSIL, HSIL, and Cancer. The x-axis represents the subjects, while the y-axis represents the Log$_{10}$ sMICA concentrations. The data points indicate varying levels of sMICA among the subjects.
Figure 2

A. Percentage of NKG2D-expressing cells by subjects (Healthy, LSIL, HSIL, Carcinoma).

B. Percentage of NKG2D-expressing cells by subjects (Healthy, LSIL, HSIL, Carcinoma).
Figure 3

Percentage of NKG2D-expressing T cells

Log10 sMICA (pg/mL)

$(r = -0.359; \text{Pearson's rho } p = 0.015)$
Healthy LSIL HSIL Carcinoma

NK cells

T cells

NKG2D expression

Figure 4
Additional files provided with this submission:

Additional file 1: table 1.pdf, 13K
Additional file 2: table 2.pdf, 11K
http://www.biomedcentral.com/imedia/1859431625159097/supp2.pdf