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Tumor-derived microvesicles in sera of patients with head and neck cancer and their role in tumor progression

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Abstract

Background: Tumor-derived membranous vesicles (MV) isolated from HNSCC patients' sera induce apoptosis of activated CD8⁺ T cells. We tested if MV molecular profile and activity correlate with disease progression.

Methods: CD8⁺ Jurkat cells were incubated with MAGE 3/6⁺, FasL⁺, MHC class I⁺ MV isolated from sera of 60 HNSCC patients and 25 normal controls (NC) by exclusion chromatography and ultracentrifugation. Z-VAD-FITC binding to Jurkat was measured and correlated with clinical data.

Results: MV from patients' but not from NC sera induced Jurkat cell apoptosis. 45% T cells+MV from N₁–N₃ patients were apoptotic vs. 18% T cells+MV from N₀ patients (p<0.008). MV from patients with **active disease (AD)** expressed higher FasL levels than MV from patients with **no evident disease (NED)** or NC (p≤0.01).

Conclusion: MAGE 3/6⁺, FasL⁺ and MHCI⁺ MV in sera of patients induced T-cell apoptosis which correlated with disease activity and the presence of LN metastases.

Keywords

tumor-derived microvesicles (MV); apoptosis; immune suppression; FasL expression; HNSCC

Introduction

The immunobiology of head and neck cancer (HNC) is strongly influenced by interactions established between the host immune system and the tumor.¹ Relative to other human solid tumors, HNC are highly immunosuppressive and often escape from immune recognition by down-regulation of key surface antigens.² Moreover, this cancer can actively corrupt the host anti-tumor immune responses via several distinct mechanisms.³ Evidence has accumulated that in patients with cancer, circulating CD8⁺ effector T lymphocytes are susceptible to spontaneous apoptosis, i.e., they are destined to die, leading to a paucity of lymphocytes responsible for anti-tumor immunity.⁴ We have been investigating

mechanisms responsible for this rapid elimination of T-cell subsets in the peripheral circulation of patients with cancer.⁵ Recently, we have observed that small (50-100 nm) membranous vesicles (MV) present in sera of patients with HNSCC are biologically active and might be responsible for the demise of activated CD8⁺ T cells in the peripheral circulation.⁶

MV originate from multivesicular bodies in a broad spectrum of epithelial and hematopoietic cells. Initially described as MV released from reticulocytes,⁷ they were also shown to be secreted by tumor cell lines.⁸ More recently, MV were reported to be secreted by dendritic cells,⁹ T cells,¹⁰ B cells¹¹ and tumor cells of different origins.¹²⁻¹³ The placenta-derived MV have also been described.¹⁴ MV vary in size from 50 to 100 nm (i.e., are the size of viruses) and are formed by inverse membrane budding into the lumen of the endocytic compartment. Following fusion of multivesicular bodies with the plasma membrane, MV are released into extracellular space.¹⁵ MV express a variety of known membrane-associated molecules which mirror the surface membrane profile of the cells from which they originate. MV derived from different cell types might have distinct biologic activities and may have opposing effects on immune responses. For example, dendritic cell (DC)-derived MV are equipped with functional MHC Class I and II molecules and, consequently, tend to promote immune responses. DC-derived MV have been used for immunotherapy of cancer in mice and were shown to induce tumor-rejection.^{9,17} Clinical studies with DC-derived MV are currently ongoing, although preliminary results are not encouraging.^{18,19} In contrast to DC-derived MV, those derived from tumors express death ligands, including FasL and TRAIL. Activated CD8⁺ T cells co-incubated with these MV undergo apoptosis which positively correlates with the level of FasL expression on MV.^{6,20}

Although fresh tumor cells and tumor cell lines release MV able to induce death of activated CD8⁺ T cells, the origin of MV present in sera of cancer patients is presently unknown. The ability of MV to eliminate circulating effector T cells *in vivo* is also not yet confirmed. Data in the literature suggest that in addition to their effects on T cells, MV adversely affect functions of DC,²¹ which are the major antigen-presenting cells (APC) and which have been reported to be functionally disabled in patients with HNSCC.²²⁻²³ Because tumor-derived MV might facilitate tumor escape by inducing immune dysfunction, we have focused on linking their molecular profile and biologic activity to tumor progression in HNSCC patients.

Materials & Methods

Patients and normal controls

This exploratory study included 60 patients with diagnosis of HNSCC. Thirty three patients were seen at the Outpatient Otolaryngology Clinic at the University of Lubeck Medical Center, Lubeck, Germany and 27 patients at the Outpatient Otolaryngology Clinic, University of Mainz Medical Center, Mainz, Germany. Also, 25 NC were recruited at the University of Pittsburgh Medical Center, Pittsburgh, PA, USA and included in this study. All patients and NC signed informed consent forms approved by the respective University Institutional Review Boards or Ethical Committees. Sera for isolation of MV (about 10mL) were obtained from venous blood of all subjects and stored at -80°C until use. The radiotherapy or radiochemotherapy, if given, was terminated at least 12 weeks *prior to the time of phlebotomy* to obtain blood for this study. Clinicopathological data of the patients included in this study are shown in Table 1.

Cell lines

Jurkat cells, originally established from a T cell leukemia patient in 1977 (Schneider, U et al, 1977), were obtained from American Tissue Culture Collection (Manassas, VA) and stably transfected with the gene encoding CD8 receptor (courtesy of Dr. H. Rabinowich, University of Pittsburgh). CD8⁺ Jurkat cells were cultured in complete medium consisting of RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin, 100 µg/mL streptomycin and L-glutamine (2mM/L). RPMI 1640 medium was used for washing cells. All reagents were purchased from Gibco/Invitrogen, Grand Island, NY.

PCI-13 is a human squamous cell carcinomas of the head and neck (HNSCC) cell line established from a primary tumor and maintained in our laboratory as previously described. 24 The parental cell line (PCI-13p) was transfected with the gene encoding human FasL obtained from Dr. S. Nagata (Osaka Biosciences Institute, Osaka, Japan), using a retroviral vector as previously described. 25 MV isolated from the culture supernatant (SN) of transfected PCI-13 cells were used as positive controls in all experiments. The tumor cell line was maintained in DMEM medium supplemented with 10% FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin and L-glutamine (2mM/L) at 37°C in an atmosphere of 5% CO₂ in air.

The cell lines were routinely tested for mycoplasma and found to be negative.

MV isolation

MV were isolated from sera of patients and NC or from cell culture supernatants (SN) using exclusion chromatography and ultracentrifugation, as previously described. 6,20 Briefly, SN of the FasL transduced PCI-13 cell line was concentrated 10x using Centriprep YM-50 centrifugal filter devices (Millipore, Billerica, MA). Bio-Gel A50m columns (2.5 × 45cm, purchased from Bio-Rad Laboratories, Hercules, CA) were packed with Sepharose 2B (Amersham Biosciences, Piscataway, NJ) and equilibrated with PBS. Next, 10mL aliquots of sera or concentrated SN were applied to the columns and eluted with PBS. The protein content was monitored by measuring absorbance at 280 nm, and material in the void volume peak, containing proteins of >50 million kDa was found to be in the fraction of 10- 28mL. Thus, three fractions 9mL each were collected, and the first fraction was discarded. The second and third fractions were combined, placed in a Beckman Optiseal Centrifuge Tubes and centrifuged in a Beckman Optima LE-80K Ultracentrifuge (Beckman Coulter) at 100,000g for 3h at 4°C. The pelleted membrane fragments were resuspended in 500 µL volume PBS and analyzed for proteins in a Lowry microassays 26 using reagents purchased from Bio-Rad Laboratories.

Western Blot Assays

Isolated MV were characterized for expression of FasL, MAGE 3/6 and soluble MHC class I molecules (sMHC I). Each MV fraction equivalent to 25 µg of protein was prepared with lysis buffer containing Halt™ Protease inhibitor (Pierce, Rockford, IL) and loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked with 5% fat-free milk in TBST (0.05% Tween 20 in Tris-buffered saline). Next, protein-loaded membranes were incubated overnight at 4°C with either anti-FasL antibody (Ab-3; Oncogene, Cambridge, MA), anti-MAGE 3/6 antibody (kindly provided by Dr. Spagnoli, Basel, Switzerland), or anti-MHC I antibody (clone: HC-10, a generous gift from Dr. Ferrone, Buffalo, NY). The bound antibodies were immunodetected using conjugated rabbit anti-human polyclonal antibody to anti-FasL Ab or anti-sMHC1 Ab, each with a concentration at 5 µg/mL (Alpha Diagnostics Inc., San Antonio, TX), or conjugated mouse anti-human monoclonal antibody to anti-MAGE 3/6 antibody, followed

by incubation with HRP-conjugated goat anti-rabbit Ig or anti-mouse (concentration at 10 μ g/mL) (Pierce). Protein bands on immunoblots were detected by enhanced chemiluminescence (Pierce), followed by autoradiography. Equal protein loading was confirmed by using anti- β -actin Ab (clone: AC-15; Sigma, St. Louise, MO). Proteins were semi-quantified by computerized densitometric analysis (Molecular Dynamics, Sunnyvale, CA).

Apoptosis assay

To assess the level of apoptosis in CD8⁺ Jurkat cells co-incubated with isolated MV, cells were labeled with CaspACETM FITC-VAD-FMK In Situ Marker (Promega, Madison, WI). This reagent is a FITC conjugate of the cell permeable pan-caspase inhibitor VAD-FMK which freely enters the cell, binds to activated caspases and thus allows for quantification of caspase levels by flow cytometry. The staining was performed according to a standard protocol provided by the manufacturer.

Jurkat cells were co-incubated for 6h with 250 μ g of isolated MV at 37°C and 5% CO₂ in air. As a positive control, Jurkat cells were incubated at 56°C for 30 min. Alternatively, Jurkat cells were incubated with anti-Fas mAb (CH-11Ab purchased from Upstate Biotechnology, Lake Placid, NY) at 250ng/mL or the respective isotype control. Still another positive control consisted of Jurkat cells co-incubated with 250 μ g MV isolated from FasL⁺ PCI-13 cell culture supernatants. Negative controls included MV fractions obtained from sera of NC.

After co-incubation with MV, Jurkat cells were harvested (at least 200,000 cells/tube), washed twice in staining buffer, consisting of PBS and containing 0.1% (w/v) BSA and 0.1% (w/v) NaN₃. Cells were stained for flow cytometry as previously described.²⁷ Briefly, for surface staining, cells were incubated with the pre-determined optimal dilutions of each mAb for 25 min at 4°C in the dark, washed twice with staining buffer and finally fixed by resuspending them in 2% (v/v) paraformaldehyde (PFA) in PBS. The following Abs were used for surface staining: anti-CD3-ECD, anti-CD8-PC5 and respective isotype control IgG₁, all purchased from Beckman Coulter, Miami, FL, USA. The cells were stored at 4°C in the dark until acquisition on the flow cytometer. All mAbs used were pre-titred on fresh PBMC to determine their optimal working dilutions.

Flow cytometry

Flow cytometry was performed using a FACScan flow cytometer (Beckman Coulter) equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were initially restricted to a small lymphocyte gate as determined by the characteristic forward and side scatter properties of lymphocytes. For analysis, 10⁵ cells were acquired, and the analysis gates were restricted to the CD3⁺CD8⁺ T-cell subset. Data were analyzed using Coulter EXPO 32v1.2 analysis software.

Transmission Electron Microscopy

MV were visualized using transmission electron microscopy. After centrifugation, the pellets were fixed in 2.5% (w/v) glutaraldehyde in PBS, dehydrated and embedded in Epon. 65nm thin sections were cut and stained with uranyl acetate and Reynold's lead citrate. The sections were examined in a Jeol 1210 transmission electron microscope at the Center for Biologic Imaging at the University of Pittsburgh School of Medicine.

Statistical Analysis

Statistical analysis was performed using Mann-Whitney U test for unpaired comparisons to analyze for differences between patients with HNSCC and normal controls. Associations

between levels of FasL, **MHCI** or MAGE 3/6 in microvesicle fractions and the disease stage or nodal involvement were analyzed using a χ^2 and Cochran-Armitage tests. A p value lower than 0.05 was considered to be statistically significant.

Results

Characteristics of patients in the study

The two cohorts of HNSCC patients were combined for this study to gain statistical power based on initial demographic and pathologic descriptors. The two groups were similar for age, gender, T stage and N stage. However, they differed in disease status, as the Lubeck cohort included 11 patients with no evident disease (NED) following oncologic therapy, while all patients in the Mainz cohort had active disease (AD). This sampling was intentional, as we wished to compare patients with NED vs. AD for the molecular profile and biologic activity of MV isolated from their sera. The patient cohorts recruited for this study represented a typical cross-section of HNSCC population. All patients ($n=60$) had histologically-proven squamous cell carcinoma in the oral cavity ($n=47$) or larynx ($n=13$). As shown in Table 1, 49 patients had an AD at the time of phlebotomy, and 11 patients treated with oncologic therapy were NED. Among the AD patients, 35 had a primary disease and 14 had a recurrent disease. Fifteen patients with an early disease (UICC I and II stages) and 45 patients with advanced disease (UICC III and IV stages) were included. Twenty eight tumors were pathologically classified at surgery as T4. Local metastases (N_{1-3}) were evident in 32 patients, and 6 patients had distant metastases (M_1). At the time of phlebotomy, the patients were not on therapy.

MV in sera of the patients and normal controls

MV were isolated from all serum specimens obtained from the patients or NC in the UPCI laboratory, using the same isolation method. The protein content of MV isolated from patients' sera was significantly higher than that of MV isolated from sera of NC ($p=0.001$). The MV fractions contained $12\mu\text{g} \pm 3.6$ (mean $\pm$ SD) protein/10mL serum in NC and $65\mu\text{g} \pm 62$ protein/10mL serum in HNSCC patients (Figure 1A). The presence of MV in the sera of HNSCC or in supernatants (SN) of HNSCC cell lines was confirmed by electron microscopy using isolated MV fractions. Sera obtained from NC contained no MV, while MV isolated from SN of FasL+ PCI-13 cells were similar in size to those present in sera of the HNSCC patients (Figures 1B, 1C and 1D).

Molecular profiles of MV in HNSCC patients and normal controls

We next determined the molecular profile of MV isolated from different samples. As shown in Figure 2A, the profile of MV isolated from sera of HNSCC patients was distinct from that of MV isolated from sera of NC. FasL was detected in the majority of patients' MV, but not in MV obtained from NC. MAGE 3/6 was present in patients' MV but not those in NC (Figure 2B). Also, levels of surface protein expression varied among the MV fractions obtained from different patients. The MHC class I molecule was expressed in MV derived from all individuals, including NC, but its expression level was significantly greater in MV from patients than those obtained from NC (Figure 2B). Expression of MAGE 3/6 could be used as a marker of MV derived from tumor cells, because we previously determined that MAGE 3/6 is detectable by immunostaining in all HNSCC specimens ($n=10$) and cell lines ($n=5$) we examined to date (unpublished data). MAGE 3/6 is not detectable in normal cells or tissues and is considered a tumor antigen. Western blot analysis showed that MV isolated from sera of healthy individuals were negative for MAGE 3/6, whereas those isolated from 61% of patients with HNSCC were positive.

Molecular profiles of **MV** and disease activity

Expression levels of MHC class I molecules and FasL in MV as determined by immunoblots were next correlated with disease activity (AD vs. NED). Expression of MHC class I molecules was relatively low in MV obtained from the patients with AD. Inversely, MV derived from sera of patients with NED showed higher levels of MHC class I expression ($p < 0.02$). MV isolated from sera of all NC expressed MHC class I molecules at a very low level (Figure 3A). More striking, expression levels of FasL on MV isolated from sera of AD patients were significantly higher ($p < 0.02$) than those in MV of patients with NED or NC (Figure 3B). As shown in Figure 3C, relative FasL expression was semiquantified on MV isolated from sera of AD patients with a mean of 497 ± 350 (SD) relative units (U) vs. $311 \text{U} \pm 120$ on MV isolated from NED patients and $3 \text{U} \pm 10$ on MV isolated from normal controls ($p < 0.01$). In aggregate, these results suggest that MV molecular profiles might be informative regarding disease activity in patients with HNSCC.

MV derived from sera of HNSCC patients induce apoptosis in **Jurkat** cells

In agreement with our previous data,⁶ MV isolated from sera of patients with HNSCC induced apoptosis in CD8^+ Jurkat cells. In this study, we measured pan-caspase activity in lymphocytes using a Z-VAD-binding fluorescence assay and MV isolated from FasL-transduced PCI-13 cells as well as CH-11Ab as positive controls. Following 6h co-incubation of CD8^+ Jurkat cells with MV isolated from patients' sera, up-regulated caspase activity was observed, as documented by elevated Z-VAD binding (Figure 4). MV obtained from sera of NC induced little pan-caspase activity in Jurkat cells. In contrast, much higher, although variable, levels of pancaspase activity were observed with MV isolated from patients' sera. When we related the levels of Z-VAD binding in Jurkat cells to clinical or pathologic endpoints, a significant association emerged between the presence of local metastases and the ability of MV to induce T-cell apoptosis. MV isolated from patients with local metastases induced pan-caspase activity in a significantly higher proportion of CD8^+ Jurkat cells relative to MV obtained from patients who were N_0 (Figures 4A and B).

In addition, MV derived from sera of patients with a large tumor mass (T3/T4) induced a higher level of Z-VAD-binding compared to those obtained from patients with an early stage of disease (T1/T2). In 72% of cases, MV isolated from patients with T3/T4 tumors **induced pancaspase activity in** Jurkat cells vs. 58% when MV isolated from sera of patients with T1/T2 tumors were used. This difference with a p-value of 0.06 indicates that MV biologic activity might be related to the tumor burden (Figure 4C).

Discussion

The hypothesis tested in this study was that tumor-derived MV present in sera of patients with cancer are capable of inducing effector CD8^+ T lymphocyte apoptosis. We hypothesized that circulating MV secreted by the tumor may be responsible for inducing tumor-associated immune suppression, thus contributing to tumor escape. Additionally, earlier evidence suggested that tumor-derived MV might serve as biomarkers of disease activity and progression in patients with cancer.⁶ To confirm and extend these previous results, we have isolated and characterized MV from a larger cohort of patients with HNSCC. Aside from providing a more robust statistical analysis correlating the presence of MV in cancer patients' sera and disease, the current study allows for a better definition of the molecular profile of tumor-derived MV.

The ability of tumor cells to interfere with the host immune system has been primarily linked to the release and the accumulation of soluble immunosuppressive factors. While such factors act locally in the tumor microenvironment, it has been difficult to visualize how

they could modulate functions of immune cells, **inducing multiple defects in lymphocytes** at sites distant from the tumor. **Nevertheless, such defects are** detectable in patients with cancer.^{3,5} **In this context**, MV could provide a transporter system for bioactive molecules, which could effectively deliver tolerogenic signals from the tumor site to distant lymphoid compartments, such as regional lymph nodes,²⁸ where the immune activation takes place. In this scenario, tumor-derived MV are visualized as vehicles capable of distributing immunosuppressive signals and contributing to the tumor escape.

Because the tumor capability to escape from the immune control indicates an aggressive disease, we thought that the MV content and their molecular profile could be linked to disease activity and progression in HNSCC. However, tumor-derived MV represent only a small fraction of MV present in sera of patients with cancer. A variety of activated cells of different origin and distinct differentiation stage also release MV. Therefore, a molecular marker which could identify tumor-derived MV in sera is needed. We have depended on expression of MAGE 3/6 previously shown by us to be present in HNSCC, albeit as an intracytoplasmic cell component²⁹ to identify tumor-derived MV. Indeed, 61% of HNSCC patients had in the serum MV containing MAGE 3/6. Although this peptide might not be useful for isolation of tumor-derived MV from patients' sera, as it may not be present on the surface of MV, in this study, it proved to be useful for confirming the tumor origin of the isolated MV.

We observed that MAGE 3/6⁺ MV also expressed higher levels of FasL than MV negative for MAGE3/6. The presence of membrane-bound FasL in MV is important for their ability to induce apoptosis of CD95⁺ activated T lymphocytes.⁶ We have previously shown that effector CD8⁺ T cells in the circulation of HNSCC patients express high levels of CD95 and no FasL.⁶ Thus, these CD95⁺ circulating T cells are sensitive to apoptosis induced by FasL⁺ MV. While in this study, we measured pan-caspase activation in lieu of apoptosis by MV, we have previously demonstrated that FasL⁺ MV can and do induce apoptosis of CD8⁺ effector cells.⁶ We, therefore, equate the flow cytometry evidence for pan-caspase activation with apoptosis of CD95⁺ T lymphocytes.

Only MV isolated from HNSCC patients' sera induced T-cell apoptosis. Further, the level of measurable MV-induced apoptosis was related to disease activity, lymph node involvement and tumor stage. These parameters are well recognized markers of prognosis and tumor progression in HNSCC.³⁰ Therefore, it appears that the presence and molecular content of serum MV in patients with cancer might serve as biomarkers of prognosis in this disease. To what extent this biomarker will live up to expectations is not clear. MV isolation from sera of patients is laborious, and without a marker like MART 3/6 to confirm their tumor origin, it is not clear that biologic activities of MV observed *ex vivo* can be directly linked to the presence in sera of tumor-derived MV.

Our study is a preliminary report suggesting that additional studies should be undertaken to explore the possibility that serum MV in patients with HNSCC might serve as biomarkers of disease.³¹ Obviously, Western blots are not the state-of-the-art methods for the definition of MV molecular profiles. Today, methodologies utilizing proteomics are widely available and when applied to MV analysis might be useful for confirming the clinical and prognostic value of MV in patients' body fluids.

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Figure 1. Microvesicles (MV) present in sera of HNSCC patients and normal controls
 In **A**, protein content/10mL of serum in HNSCC patients and **normal controls (NC)**. **Microvesicles** were isolated by exclusion chromatography and ultracentrifugation, as described in Materials and Methods.
 In **B-D**, transmission electron microscopy of negatively stained **microvesicles** fractionated from supernatants of FasL⁺ PCI-13 cells (**B**); from serum of a representative **normal control** (**C**); and from serum of a representative patient with HNSCC (**D**). The bar indicates 100nm.

Figure 2. The molecular profile of microvesicles isolated from sera

In **A**, Western blot analysis of microvesicles for expression of FasL, MAGE 3/6 and MHC class I (MHC1) in fractions purified from sera of normal controls (NC), HNSCC patients (P1 – P5) and supernatants of a FasL⁺ PCI-13 cell line. Representative blots are shown. The numbers on the right indicate molecular weights of the detected proteins. In **B**, box-plots display distribution of results from semiquantitative immunoblots analysis showing a median as well as the first & third quartile, including outliers.

Figure 3. MHC class I and FasL expression levels in microvesicles isolated from sera of HNSCC patients with active disease, no evident disease and normal controls

In **A**, the percent of patients with serum-derived microvesicles which show low or high levels of MHC class 1 expression. Results are shown for patients with **active disease** (AD, n=48) or **no evident disease** (NED, n=11). In **B**, the percent of patients with serum-derived microvesicles which show high or low levels of the membrane form (42kDa) of FasL. In **C**, levels of FasL expression semiquantified based on densitometry readings and presented in relative units for microvesicles obtained from patients with active disease (AD), no evident disease (NED) or normal controls (NC). The p-values indicate a significant difference between AD and NED patients. Corresponding fractions of sera obtained from NC (in A or B) contained none or low levels of MHC I or FasL.

B

C

Figure 4. Pancaspase activation in CD8⁺ Jurkat cells co-incubated with microvesicles derived from sera of patients with HNSCC

In **A**, Z-VAD FMK binding to CD8⁺ Jurkat cells after treatment with heat or co-incubation with microvesicles of different origin, or with CH-11 antibody used as a positive control. Cells were co-incubated with microvesicles for 6h at 37°C in 5% CO₂ in air, or treated for 30min at 56°C. Z-VAD-binding in Jurkat cells indicates pan-caspase activation which is taken as a sign of apoptosis. *Upper row*: in control experiments, caspase activation after the treatment with 56°C, FasL Ab, or microvesicles isolated from FasL⁺ PCI-13 SN. *Lower row*: representative blots show Z-VAD-binding in Jurkat cells after incubation with microvesicles from normal controls or a representative HNSCC patient without (N₀) or

with (N_1) local metastases. In **B**, box plots summarize biologic activity of microvesicles derived from patients with (n=32) or without (n=28) lymph node metastase. MV biologic activity was determined measuring pan-caspase activation in CD8⁺ Jurkat cells after co-incubation with microvesicles for 6h. In **C**, box plots show biologic activity of microvesicles derived from patients with T1/2 (n=24) vs. T3/4 (n=36).

Table 1

Clinicopathologic characteristics of the patients with HNSCC

	n
Patients	60
Age (years)	
mean	59 ±10.71
range	33 – 84
Sex	
male	44
female	16
Disease activity	
AD ¹	49
no previous treatment	35
recurrence	14
NED ¹	11
Tumor Grade	
1	6
2	8
3	13
4	1
n/a	27
Tumor stage	
T1	15
T2	9
T3	8
T4	28
Nodal status	
N0	28
N1	5
N2	23
N3	4
Metastasis	
M0	54
M1	6
UICC stage²	
I	11
II	4
III	8
IV	37

¹ AD = Active Disease; NED = no evident disease² UICC = Union Internationale Contre le Cancer