Detection of micrometastatic tumor cells in head and neck squamous cell carcinoma

A possible predictor of recurrences?

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ABSTRACT

Objective: To evaluate the presence of micrometastatic tumor cells in the peripheral blood samples of the patients with head and neck squamous cell carcinoma (HNSCC) and to determine whether the presence of micrometastatic cells had any biological relevance in terms of local recurrences or metastasis during a follow-up period of 3 years.

Methods: We included 21 consecutive patients with untreated primary HNSCC admitted to the Ear Nose and Throat Department of Akdeniz University Medical School, Antalya, Turkey between February and October 2002. Squamous carcinoma cells in peripheral blood samples of these patients prior to surgery were detected via a magnetic cell separation technique using anti-epithelial cell adhesion molecule antibody, and thereafter evaluated by light microscopy with hematoxylin and eosin staining.

Results: Seven out of 21 patients showed squamous carcinoma cells in peripheral blood samples. Patients with stage III and IV tumors were nearly 5 times more likely to show micrometastatic cells compared with those with stage I and II tumors (6/12 versus 1/9). During the follow-up, 2 patients out of 7 with micrometastasis had recurrences. None in the micrometastasis negative group relapsed.

Conclusion: We suggest that HNSCC patients with detectable tumor cells in peripheral blood represent a subset of patients who should be followed up more closely for possible recurrences.

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Head and neck cancers are among the major health problems in the world leading to hundreds of thousands of deaths each year.[1] Head and neck squamous cell carcinoma (HNSCC) represents nearly 95% of head and neck tumors.[2] Approximately one third of the patients present with early-stage disease (stage I–II), whereas two-thirds of patients present with advanced-stage disease (stage III-IV).[3] In a large series of patients, development of distant metastases was observed in 15-25% and generally occurred within 2 years after loco-regional treatment.[4,5] However, in subgroups of patients with advanced disease, the incidence of distant metastasis may be as high as 50%.[3,4] Although the metastatic process is incompletely understood, it is known to involve separation of a group of clonogenic cells from the primary, entry into and transportation by lymphatic or vascular channels, arrest into the target organ, and proliferation.[4,6,7] Currently the presence of metastatic spread to lymph nodes in the neck is accepted to be the most important prognostic indicator for relapse of HNSCC, both locoregionally and at distant sites. Therefore, staging systems are very helpful to predict the patients at risk of recurrences. However, there is still a need for additional methods to more precisely identify the patients at risk. Micrometastasis is defined as the microscopic (<2 mm) deposit of malignant cells distinct from the primary lesion.[6] Alternative methods to detect micrometastasis as a surrogate marker of dissemination have been extensively studied during the last decade in the bone marrow and blood samples of patients with HNSCC.[2-6,8,9] In recent years, increasing attention has been directed towards the use of monoclonal antibodies against cytokeratin, E48 and p53 to detect

micrometastatic carcinoma cells.[2,8,9] Also molecular biological techniques, such as reverse transcription-polymerase chain reaction (RT-PCR), which enables subsequent amplification of specific messenger, have improved the detection rate of occult metastasis.[3,5] Immunomagnetic separation is one of the several methods used for detecting carcinoma cells in the peripheral blood of cancer patients. The system allows the selection of cells of interest (in this case squamous carcinoma cells) from a large sample of mononuclear cells. Magnetizable microbeads covered with a monoclonal antibodies specific to a certain antigen of carcinoma cells are used to identify these cells. The epithelial cell adhesion molecule (EpCAM; human epithelial antigen; CD326) is a type I transmembrane glycoprotein expressed in most normal epithelial, but is absent from squamous stratified epithelia.[10,11] However, de novo expression occurs on squamous carcinoma cells.[10,11] The EpCAM expression has previously been found to correlate with cell proliferation, dedifferentiation and tumor progression.[11,12]

The main aim of this study was to evaluate the presence of micrometastatic tumor cells in the peripheral blood samples of the patients with HNSCC via a magnetic cell separation technique using anti-EpCAM antibody. Our secondary aim was to determine whether the presence of micrometastatic cells had any biological relevance in terms of local recurrences or metastasis during short-term follow-up (3 years).

Methods. Twenty-one consecutive patients with untreated primary HNSCC admitted for treatment to the Ear Nose and Throat Department of Akdeniz University Medical School, Antalya, Turkey were enrolled in the study between February and October 2002. Age, gender, history of alcohol or cigarette consumption, localization of the tumor, stage of the tumor, tumor necrosis marker classification, modes of treatment, and outcomes for the first 3 years following the surgery were noted for each patient. Prior to surgery, mononuclear cells were isolated from 30 ml of venous blood samples collected to ethylene diamine triacetic acid (EDTA) coated tubes by density gradient centrifugation using Ficoll. After being washed twice by centrifugation through phosphate buffered saline (PBS) containing 2 mM EDTA, one more washing step was carried out with PBS containing 2 mM EDTA plus 0.5% bovine serum albumin. Then, the supernatant was removed and cell pellet was resuspended with PBS buffer in a final volume of 300 μl per 5 x 10^7 total cells. After blocking nonspecific binding of antibody coated microbeads by FcR blocking reagent (100 μl per 5 x 10^7 cells), 100 μl of anti-human epithelial antigen conjugated microbeads (HEA MicroBeads; Miltenyi Biotech, Inc, Auburn, Calif) were added making up the final labeling volume of 500 μl per 5 x 10^7 cells. Cells were incubated in this suspension with microbeads for 30 minutes at 8^OC. After incubation, the cells were washed by buffer to remove unbound beads and centrifuged at 300 g for 10 minutes. The supernatant was then removed and the cell pellet was resuspended in 500 μl of buffer per 10^8 cells. The separation column was placed in the magnetic field of an appropriate magnetic activated cell sorting (MACS) separator and washed with PBS buffer. The cells were passed through PBS wetted 30 μm nylon mesh to remove any clumps. Cell suspension in buffer was applied onto the column along the separator for 3 minutes at room temperature to bind the positive cells and to let the negative cells pass through. The column was removed from the separator after rinsing with buffer. The column was then placed on a suitable collection tube, 3 ml of PBS buffer containing 2 mM EDTA and 0.5% bovine serum albumin was applied onto the column and using the plunger supplied with the column, retained cells were firmly flushed out. Cytopreperations were fixed in 4% formaldehyde fixative and 10-20 cytopreparations were made by applying to a single funnel (Shandon Cytospin 4). Preparations were stained with hematoxylin and eosin and all slides were viewed with an Olympus CH40 microscope. All fields of all preparations were evaluated by 2 pathologists blinded to each other. Atypical cells with an abnormal morphology were detected. All atypical cells observed by the pathologists with typical characteristics of squamous cell carcinoma were noted. Mononuclear cell clusters consisting of atypical squamous cells with a big nucleus and a large keratinized cytoplasm were regarded as tumoral cells.

Results. The clinicopathological characteristics of each patient are presented in **Table 1**. Out of 21 patients, 19 were males (90%). Mean age of the patients was 60.1 (SD ± 9.4). One patient had lip, 5 tongue, one buccal mucosa, 8 supraglottic, and 6 glottic squamous cell carcinomas. All were heavy smokers, while 16 consumed moderate or heavy amounts of alcohol regularly. Two of the patients had stage I, 7 stage II, 10 stage III, and 2 stage IV tumors. Micrometastasis was observed in 7 patients (33%) of whom one was stage I, none were stage II, 4 were stage III, and 2 were stage IV. The number of patients at each stage, number of micrometastasis positive cases according to stage, and percentage of patients with micrometastasis are summarized in **Table 2**. Micrometastasis was observed in one of tongue (1/5), 2 of glottic (2/6), and 4 of supraglottic (4/8) tumors (**Figure 1** shows one of the carcinoma cells detected). The 2 pathologists fully agreed on these 7 cases. During the 3 years follow-up one patient with stage III glottic

Table 1 - Characteristics and outcomes of patients.

Patient	Gender	Age	Cancer location	TNM class	Stage	Tobacco (pack/day)	Alcohol use	Follow-up results (3 years)
1*	M	62	Glottic area	T3N0M0	III	1	Heavy	Contralateral lymph node
2	M	65	Supraglottic area	T2N0M0	II	1	Moderate	Event free
3	M	55	Tongue	T2N0M0	II	2	Heavy	Event free
4	M	53	Lip	T2N0M0	II	1	Occasional	Event free
5	M	70	Supraglottic area	T2N0M0	II	1	Moderate	Event free
6	M	52	Buccal mukosa	T3N0M0	III	2	Moderate	Event free
7*	M	60	Supraglottic area	T3N1M0	III	1	Moderate	Event free
8*	M	56	Glottic area	T1bN0M0	I	1	Moderate	Event free
9	M	45	Supraglottic area	T3N1M0	III	1	Occasional	Event free
10	M	58	Glottic area	T3N0M0	III	2	Moderate	Event free
11*	M	70	Supraglottic area	T4N1M0	IV	1	Heavy	Event free
12	M	75	Tongue	T2N1M0	III	1	Moderate	Event free
13	F	64	Tongue	T2N0M0	II	1	None	Event free
14	F	61	Tongue	T3N0M0	III	1	Occasional	Event free
15	M	44	Glottic area	T2N0M0	II	1	Moderate	Event free
16*	M	62	Supraglottic area	T4N1M0	IV	2	Heavy	Event free
17	M	70	Glottic area	T1N0M0	I	1	Moderate	Event free
18*	M	51	Tongue	T3N1M0	III	2	Moderate	Local recurrence
19	M	65	Supraglottic area	T3N1M0	III	1	Occasional	Event free
20	M	47	Glottic area	T2NM0	II	1	Moderate	Event free
21*	M	77	Supraglottic area	T3N0M	III	1	Moderate	Event free

*Patient has detectable tumor cells in peripheral blood, one pack - 20 cigarettes, M - male, F - female, TNM - tumor, lymph node, metastasis classification

Table 2 - Total numbers and percentage of micrometastasis observed in each group according to stage at presentation.

Tumor Stage	Total number of patients	Patients with micrometastasis N (%)
Stage I	2	1 (50)
Stage II	7	0 (0)
Stage III	10	4 (40)
Stage IV	2	2 (100)
All stages	21	7 (33)

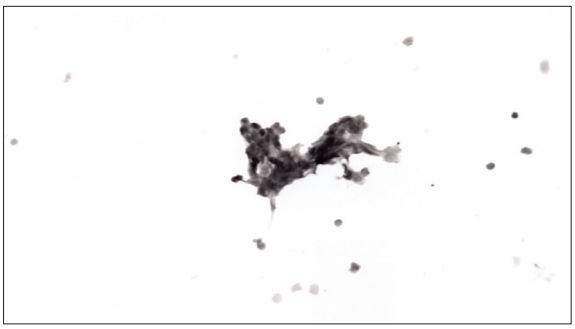

Figure 1 - Cluster of squamous tumoral cells in peripheral blood (staining: hematoxylin and eosin. Magnification: x 400).

tumor showed contralateral neck metastasis (patient 1), and another with stage III tongue cancer showed local recurrence (patient 18). Both of these patients were in the group that showed micrometastasis prior to surgery. Thus, 2 out of 7 micrometastasis positive patients showed recurrences, no other patients either in the micrometastasis positive (total of 7 patients) or negative group (total of 14 patients) showed any recurrences or metastasis during the 3 year period. Therefore, documentation of micrometastasis had 100% sensitivity, 74% specificity, 29% positive predictive value, and 100% negative predictive value for the detection of recurrences.

Discussion. We observed micrometastasis in peripheral blood of 7 out of 21 (33%) patients and followed them for a minimum period of 36 months (range 36-42). Wirtschafter et al[2] detected micrometastasis in 8 of 18 (44%) patients with HNSCC by using the same preparation technique. However, they employed a further step of alkaline phosphatase immunostaining by using an anticancer monoclonal antibody coctail (anticytokeratin type 8 and 18, TFS-2, antikeratin) to more specifically detect cancer cells. The present study is the first one in the literature employing this technique (immunomagnetic detection of HNSCC in peripheral bloods of patients by using anti-EPCAM antibody) with follow-up. The smaller percentage of micrometasis observed in our study might be due to the higher percentage of participants with stage III and IV tumors in Wirtschafter's group compared with our study (15/18 versus 12/21). Another explanation might

be false negative evaluation of the specimens. Previously a considerable inter-observer variation in the diagnosis of squamous cell carcinoma by hematoxylin-eosin staining has been reported.[13,14] Since the 2 pathologists fully agreed on all samples, we believe inter-observer variation was not a problem in this study.

Bone marrow samples have more frequently been used to detect micrometastasis in HNSCC, as well as tumors of other body sites. In HNSCC, micrometastatic tumor cell recovery rates between 11-40% have been reported according to the different techniques (such as immunocytochemistry, RT-PCR, flow cytometry) used and different antigens (such as A45/B-B3, cytokeratin 18 and 19, E48). Because not only the assays, but also the study designs differ, such inconsistency between the results of studies is expected. Gath et al,[15] using a monoclonal antibody (A45/B-B3) which recognizes specific cytokeratins expressed in HNSCC, documented micrometastasis in bone marrow samples of nearly 20% of their patients. Wollenberg et al,[16] by using monoclonal antibodies against cytokeratin 19, also immunocytochemically documented the presence of micrometastatic tumor cells in the bone marrows of patients with head and neck cancer, with an increasing rate according to the stage of the disease (26.3% versus 47.7%, for stage I and stage IV tumors). Other studies also demonstrated more frequent recovery of malignant head and neck cancers from bone marrow samples compared with peripheral blood.[3,9] By using immunocytochemistry (ICC) (with-pancytokeratin antibody) and E48 RT-PCR, Partridge et al[9] showed that bone marrow samples were 1.6-2 times more likely to show tumor cells than simultaneous blood samples in head and neck cancer patients. This discrepancy is believed to arise from entrapment of probably phenotypically different tumor cells in the bone marrow. We have observed an increase and decrease in pattern in the rate of micrometastasis according to individual stage, probably due to our limited sample size (**Figure 1**). However, when we arbitrarily grouped stage I-II tumors as "early stage tumors" and stage III-IV as "late stage tumors", we have observed nearly a 5 fold increase in micrometastasis detection rates in late stages compared with the early ones (1/9 versus 6/12), probably due to increased tumor burden in the latter. The role of tumor burden in micrometastasis detection rates is also shown by longitudinal studies where preoperative samples were found to have a higher tumor cell recovery yield compared with postoperative ones.[9]

Out of all others, the most important question remains to be answered is whether detected tumor cells either in the bone marrow or blood have any prognostic value. Different studies with average follow-up periods ranging from 17-49 months have revealed that detection of micrometastasis in bone marrow samples have prognostic value to some extent. Gath et al[15] showed that tumor relapse was 2 times more likely in patients with micrometastasis, Wollenberg's[16] results supported that micrometastasis was associated with higher local and distant metastasis as well as shorter disease free survival. Others argued that micrometastasis was a prognostic factor only in the presence of 2 or more lymph node metastasis at the time of diagnosis.[5] We observed recurrences in 2 of our patients both of whom had stage III tumors irrespective of nodal involvement at the time of initial diagnosis. These patients were from micrometastasis positive groups and no other recurrences were observed either in the micrometastasis positive or negative groups. Although longer follow-up periods are needed to more accurately detect patients with recurrences, it is well known that most cases recur during the first 2 years after the treatment. Our results showed that micrometastasis positivity had very high sensitivity (100%), and negative predictive value (100%) in determining recurrences. However, we estimates of sensitivity, specificity, positive and negative predictive values are imprecise due to our limited sample size. Although our results are insufficient to prove a very clear association, we suggest that patients with detectable tumoral cells in peripheral blood samples are more likely to recur compared with those who do not show these cells, and therefore should be followed up more carefully. Because tumorigenesis and recurrence are multifactorial processes (determined by genetic, local, immunologic, and other predisposing factors such as on going tobacco and alcohol exposure) we could not clearly depict why other patients with micrometastasis did not recur. Since EpCAM is a structural protein, it might be argued that its presence does not prove that the detected cells are viable. They may well be dead shed cells from the tumoral tissue. Although this probability cannot be completely excluded, due to absence of characteristics of non-viable cells on pathological examination, this explanation is unlikely.

In conclusion, we suggest that HNSCC patients with detectable tumor cells in peripheral blood represent a subset of patients who should be followed up more closely for possible recurrence. It could also be noteworthy to follow-up a cohort of patients who were initially micrometastasis negative and turn out to be positive during follow-up, in order to review the clinical significance of detected cells. There is still a need for larger studies (ideally that include control groups such as healthy individuals or patients with nonmalignant conditions, namely; polyps), and further proof to change treatment options for those who show micrometastasis.

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