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Sensitive Detection of Squamous Cells in Bone Marrow and Blood of Head and Neck Cancer Patients by E48 Reverse Transcriptase-Polymerase Chain Reaction

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Abstract

In previous studies, we described the selective reactivity of monoclonal antibody E48 with normal squamous and transitional epithelia and their malignant counterparts and the capacity of monoclonal antibody E48 for selective tumor targeting in head and neck cancer patients. Cloning of the E48 encoding cDNA and elucidation of the gene structure enabled the selection of an intron-spanning primer set for the detection of circulating tumor cells in blood and bone marrow of head and neck cancer patients. Extensive optimizations led to a reproducible reverse transcriptase-PCR assay with an internal standard for RNA quality control and an external standard for sensitivity control. In reconstruction experiments, we were able to reach a reproducible sensitivity of one single tumor cell per 7 ml of blood (2 × 10^7 nucleated cells). When applying this method to patient material, we were able to detect positive signal in 35% of the bone marrow samples (0 of 2 stage II, 0 of 4 stage III, 4 of 11 stage IV, and 4 of 6 recurrences) and 10% of the blood samples (2 of 21) of patients with squamous cell carcinoma of the head and neck. The specificity of the method was demonstrated on 29 blood and bone marrow samples of noncancer controls, which were all negative. Our study shows the feasibility of E48 reverse transcriptase-PCR for the detection of squamous cells in nonsquamous tissues.

Introduction

SCC2 represents ~90% of all head and neck cancers, with an incidence of 500,000 cases worldwide (1, 2). Moreover, SCC is also the major histological type among tumors of the lung, esophagus, cervix, and skin. About one-third of the patients with HNSCC presents with early-stage disease (stage I and II), whereas two-thirds presents with advanced disease (stages III and IV; Ref. 3). In the last 20 years, there have been clear improvements in the treatment of HNSCC, especially due to the progress in the fields of surgery and radiotherapy. Despite these improvements, the 5-year survival rates of HNSCC patients did not increase substantially during this period. It appears that, even with the currently applied methods for locoregional treatment, small undetectable tumor deposits frequently remain in the body (“minimal residual disease”), resulting in local and distant relapse within a short period after primary therapy. In patients with stage III and IV disease, who are treated in general with a combination of surgery and radiotherapy, local recurrences occur in 50–60% of the cases, whereas ~25% develop distant metastases (4). The frequency of distant metastases is even higher because autopsy studies report on an incidence of 40–57% (5–7). The lungs are the most frequent site of metastases, followed by the skeleton. Other localizations of metastatic deposits are the liver, mediastinum, skin, and brain (8, 9). It has been shown that the incidence of both local recurrences as well as distant metastases is directly related to the number of lymph node metastases (9, 10). For example, when four or more lymph nodes contained tumor, the risk of developing distant metastases was found to be ~50% (9).

Treatment failure is often accompanied by a failure in diagnosis. For example, ~50% of the patients with tumor-free margins after surgery, as defined by conventional histopathological examination, appear to have tumor cells in the surgical margins when assessed by molecular analysis using p53 mutations (11). In addition, distant relapse occurs in patients for whom there was no evidence for the presence of residual disease after primary therapy, indicating that the current methods meet limitations with respect to sensitivity.

With respect to systemic dissemination, distant metastases can be detected today only at a late stage, e.g., by computed tomography of the lungs, bone scintigraphy, or ultrasound of the liver, when they have a diameter of at least 1 cm. For patients with such large metastases, there are no curative treatment options available. However, recent developments in immunology and molecular biology make it possible to detect single tumor cells in body fluids. In recent years, several papers have been published on the detection of micrometastatic tumor cells in bone marrow and peripheral blood of patients with different types of cancer by immunocytochemistry (Ref. 12; reviewed in Refs. 13 and 14) and by RT-PCR (15–20). For these purposes, epithelial antigens such as CKs or other tumor-associated antigens are exploited for detection of disseminated tumor cells. For some tumor types, the presence of disseminated micrometastatic cells in bone marrow or peripheral blood at the time of primary...
surgery was shown to be related with early relapse and decreased survival (20–22).

Two recent studies indicate that tumor cells can be detected in the bone marrow of HNSCC patients (23, 24). Both authors used the alkaline phosphatase anti-alkaline phosphatase technique in immunocytochemistry with MAbs directed against CKs for the identification of single isolated epithelial cells in bone marrow of head and neck cancer patients. The presence of these cells at the time of primary treatment appeared to indicate a significant higher risk for development of local recurrences or distant metastases and a significant shorter disease-free interval (23, 25).

At our laboratory, we are focusing on the use of SCC-associated antigens and MAbs for detection and eradication of minimal residual HNSCC. For this purpose, we have developed MAb E48, which is selectively reactive with normal and malignant squamous and transitional epithelia (26). Moreover, MAb E48 has been tested extensively in radioimmunoscintigraphy and biodistribution studies in HNSCC patients (27, 28). Biodistribution studies and dosimetry calculations indicated the feasibility of adjuvant radioimmunotherapy for eradication of minimal residual disease after primary therapy (29).

Recently, the cDNA encoding the MAb E48 defined antigen was cloned, and the gene structure was elucidated (30, 31). Sequence analysis revealed that the E48 antigen is a M, 15,000–22,000 glycosylphosphatidylinositol-anchored membrane protein that shares 72% sequence homology with the mouse ThB antigen, a member of the murine Ly-6 family. Using this molecular information, we now describe an E48-specific RT-PCR assay for the detection of HNSCC cells in bone marrow and peripheral blood, which has been tested extensively in model seeding experiments. In addition, preliminary data on the detection of micrometastatic tumor cells in the circulation of HNSCC patients are presented.

Materials and Methods

Cell Lines and Tissue Samples. The human HNSCC cell lines UM-SCC-22A, UM-SCC-22B, UM-SCC-14C, and UM-SCC-11B, used for Northern blotting and reconstruction experiments to assess the sensitivity of the E48-specific RT-PCR procedure, were kindly provided by Dr. T. E. Carey (University of Michigan, Ann Arbor, MI). In the reconstruction experiments, HNSCC cells were micromanipulated under a Zeiss SV11 stereomicroscope, and different numbers were added to 7-ml peripheral blood samples obtained from healthy volunteers in heparin vacutubes. The viability of the HNSCC cells used for seeding was always 95%, as assessed by trypan blue exclusion.

Bone marrow aspirates and peripheral blood samples were obtained in heparin vacutubes from 28 HNSCC patients scheduled for surgical treatment and 29 noncancer controls. Bone marrow was taken under general anesthesia from the iliac crest at one side. Blood samples of HNSCC patients were taken from the arterial line, one sample just before surgery and one sample after tumor resection and before reconstruction. Blood samples of noncancer controls were obtained by aspiration of venous blood. The primary tumor and the status of the lymph nodes of the HNSCC patients were classified according to the tumor-node-metastasis system of the International Union Against Cancer. Patient characteristics are shown in Table 1. The protocol was approved by the institutional review board of the University Hospital Vrije Universiteit.

Preparation of RNA. Peripheral blood and bone marrow aspirate samples were diluted 1:1 in RPMI 1640. After centrifugation at 220 × g for 10 min, the erythrocytes in the pellet were lysed in 10 (blood) or 5 (bone marrow) ml of lysis buffer containing 160 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA (pH 7.4) and incubated at 4°C for 15 min while the solution was mixed occasionally by inverting the tube. Nucleated cells were pelleted by centrifugation at 220 × g for 10 min, washed with 10 ml of RPMI 1640, and centrifuged at 220 × g for 5 min. Cells were resuspended in 1 ml of RPMI, transferred to a 1.5-ml microcentrifuge tube, and centrifuged at 12,000 × g for 1 min. The pellet was dissolved in 1.0 ml of RNAzol-B (Campro Scientific, Veenendaal, the Netherlands) and mixed. After the addition of 100 μl of chloroform, the mixture was mixed again for 15 s and put on ice for 5 min. After centrifugation at 12,000 × g × 15 min at 4°C, the aqueous phase was transferred to another microcentrifuge tube, the RNA was precipitated by addition of an equal volume of isopropanol, and the precipitate was stored until use at −20°C. Just before use, RNA was pelleted by centrifugation at 12,000 × g for 15 min. The pellet was washed by vortexing in 70% ethanol, centrifuged at 12,000 × g for 5 min, and dissolved in 20 μl of RNase-free H2O. The amount of RNA was calculated from the absorbance at 260 nm.

Synthetic Oligonucleotides for RT-PCR. Fig. 1 shows the E48 gene structure and the location of the oligonucleotide primers E48-15 s and E48/95as, used for E48 mRNA detection. The sequence of the sense primer E48-15 s was 5′-CAGACGACATCAGAGATGGACAGACGC-3′, and that of the antisense primer E48/95as was 5′-GGGCAGACCCACAATGCTTGC-3′. Using these intron-spanning primers, we amplified a 130-bp RT-PCR product when E48 mRNA was present.

The quality of RNA was confirmed by amplification of the Abelson gene transcripts using the sense primer ABL1 (5′-TGTGATTATAAGCTAAGACCCCGAG-3′) and the antisense primer ABL3 (5′-TTCAGCCGGCAGGTACATCTGACTTGC-3′). When using these intron-spanning primers, we obtained a 185-bp product when Abelson mRNA was amplified (15). Oligonucleotides were obtained from Isogen (Maarsen, the Netherlands).

RT-PCR Method. In total, 5 μl of the total RNA sample were added to a final volume of 20 μl containing 25 pmol of E48/95as or ABL3 primer; 50 mM Tris-HCl (pH 8.3); 60 mM KC1; 30 mM MgCl2; 1 mM DTT; 0.1 mM each dCTP, dATP, dGTP, and dTTP (Boehringer Mannheim, Almere, the Netherlands); 0.7 units of avian myeloblastosis virus reverse transcriptase (Promega, Leiden, the Netherlands); and 2 units of RNasin (Promega). The solution was incubated with gentle agitation for 2 h at 42°C.

Four 5-μl aliquots were used for PCR and dispensed in a final volume of 50 μl containing 25 pmol of each sense and antisense primer (E48 or ABL), 5 mM Tris-HCl (pH 8.3), 44 mM KC1, 1.2 mM MgCl2, and 0.2 units of AmpliTaq DNA polymerase (Perkin-Elmer Nederland BV, Gouda, the Netherlands). PCR was carried out on a PTC200 DNA Engine (MJ Research; Biozym, Landgraaf, the Netherlands). Samples were denatured at 90°C for 4 min, followed by 40 cycles of 1 min at 90°C, 1 min at 65°C, 1 min at 72°C for 2 min, and, finally, 4 min at 72°C.

Southern Blotting and Hybridization. For Southern blot hybridization, 10 μl of the reaction products were run on an
Isolated from bone marrow; ND, not determined; Abl BL, Abelson signal in RNA isolated from blood; E48 BL, E48 signal in RNA isolated from blood; Abl BM, Abelson signal in RNA isolated from bone marrow; E48 BM, E48 signal in RNA isolated from bone marrow; ND, not determined; + + , strong positive; + , positive; = , negative; AL, alive; DOC, dead of other causes; DOD, dead of disease; UNK, unknown.

Numbers in parentheses indicate the number of months after removal of the tumor that status was assessed.
Sensitive Detection of Micrometastasis in Squamous Cancer

1 cell in 7 ml of blood was reached in every single experiment. In all experiments a blank control (no RNA template, depicted with 0) was added. PCR products were run on an 2% agarose gel, blotted, and hybridized with the E48 cDNA as a probe. The probe consists of nucleotides 29–772 of the E48 cDNA, as described previously (30).

Preparations in which RNA was omitted served as negative RT-PCR control, and reactions without reverse transcription were used as negative PCR control. The Abelson primer set was used to check the quality of the RNA.

In experiments with clinical material, an external control to the RNA laboratory to the amplimer laboratory. To prevent contamination of clinical samples with probe ammeters in one-way direction: from the primer laboratory to the RNA laboratory to the amplimer laboratory. To prevent contamination of clinical samples with probe amplimers, the cDNA probe was shortened by PCR and subcloning into Escherichia coli to remove the sense primer sequence. The probe consists of nucleotides 29–772 of the E48 cDNA, as described previously (30).

Preparations in which RNA was omitted served as negative RT-PCR control, and reactions without reverse transcription were used as negative PCR control. The Abelson primer set was used to check the quality of the RNA.

In experiments with clinical material, an external control corresponding to 1 and 10 cells per 7 ml of blood (5 and 50 pg of UM-SCC-22A RNA, respectively, in 5 μg of WBC RNA) was run in parallel to show that the indicated sensitivity level of 1 cell in 7 ml of blood was reached in every single experiment.

Results

E48 RT-PCR. After molecular cloning of the E48 antigen encoding cDNA and elucidation of the gene structure, we determined an RT-PCR primer set with the sense primer identical to a sequence in exon 1 and an antisense primer complementary to a sequence in exon 2 (Fig. 1). To determine the sensitivity of the assay, we seeded HNSCC cells with high E48 expression into blood samples of healthy volunteers. Initial experiments revealed that amplification of the 908-bp gene fragment occurred due to the fact that, in almost all RNA preparations, a small contamination of genomic DNA was present. Because the intron between exons 1 and 2 is only 778 bp, the gene fragment can be amplified effectively by PCR. Amplification of this genomic DNA fragment caused a dramatic reduction of the amplification of the cDNA fragment. Decreasing the denaturation temperature prevented amplification of the genomic fragment and resulted in an increase of amplification of the cDNA fragment (Fig. 2). The basis of this enhanced sensitivity is most likely the difference in the molecular weight and, thus, the melting temperature of the genomic DNA as compared to the cDNA fragment. The same method was used for a different primer set as well (CK19) but was shown to be less effective (data not shown).

Reconstruction Experiments with E48-expressing HNSCC Cells. The RT-PCR protocol with reduced denaturation temperature was used to analyze the specificity and sensitivity in model seeding experiments. One, 5, or 25 UM-SCC-22A cells were added to 7 ml of blood of healthy volunteers and assayed by E48 RT-PCR. As depicted in Fig. 3, one cell in 7 ml of blood, corresponding to ~2 × 10⁷ WBCs, could be detected in these experiments.

The initial experiments were all carried out with cell line UM-SCC-22A, a HNSCC cell line with high E48 expression. It should be noted, however, that, although most squamous cell tumors express the antigen, the expression is sometimes heterogeneous. Cell line UM-SCC-14C (negative when immunocytochemically stained with MAb E48) has a very low level of E48 transcripts, which was not detectable by Northern blotting, and by using RT-PCR, we showed that the expression is ~1000-fold lower than that of cell line UM-SCC-22A. In model seeding experiments with cell line UM-SCC-14C in blood, the detection level was about five cells in 7 ml of blood. Although the cell line is negative on Northern blot as well as by antibody staining, the level of expression is still high enough for a moderate sensitive detection (data not shown).

Reproducibility. To test the reproducibility of the RT-PCR on the level of one cell per 7 ml of blood, we performed a blinded seeding experiment. To four of eight tubes of blood, a single UM-SCC-22A cell was added. The reaction was performed in quadruplicate: two reverse transcription reactions with two PCRs each. As shown in Fig. 4, the cells in tubes 1 and 8 (from left to right) were reproducibly detected in all reactions, whereas the cells in tubes 3 and 5 are detected only in one of four reactions. The same effect was observed when four PCRs were carried out from one RT reaction (data not shown). Although the effect is relatively perpendicular, i.e., there is usually full presence or total absence of signal even after overexposure...
of the autoradiogram, we hypothesized that the basis of this difference nonetheless relies on the heterogeneous E48 expression of this cell line. PCR is an exponential technique, and small variations in the amount of cDNA template might lead to the presence or absence of signal rather than a diminished amount of signal. When the detection limit is reached (one cell), this is not reflected by a diminished amount of signal in four of four reactions but in a diminished reproducibility from four of four reactions to one of four, two of four, or three of four reactions.

From previous fluorescence-activated cell sorting experiments and immunocytochemical stainings, it is known that there is a considerable variation in E48 expression between individual UM-SCC-22A cells. Thus, the lack of reproducibility when single cells are seeded could, in fact, reflect the variation in antigen expression. This hypothesis was tested by a serial dilution experiment. The amount of RNA isolated from 7 ml of blood is usually ~20 μg, of which 5 μg is assayed. The amount of RNA in a single UM-SCC-22A cell is 17 pg, indicating that a ratio of 1 pg of UM-SCC-22A RNA diluted in 1 μg of WBC RNA roughly corresponds to one UM-SCC-22A cell per 7 ml of blood. Using this ratio, we were able to detect positive signal in a range of 0.5–15 μg of total RNA in the RT-PCR (data not shown), indicating that, over a wide range of input RNA, the one cell per 7 ml of blood detection level can be reached. In a serial dilution experiment, we added 20, 2, and 0.2 pg of UM-SCC-22A RNA to 2 μg of WBC RNA, corresponding to 10, 1, and 0.1 cell per 7 ml of blood, respectively, and performed an RT-PCR in quadruplicate (one reverse transcription reaction, four PCRs). As can be seen in Fig. 5, on the level of 10 cells per sample (20 pg), all assays were positive, whereas the level of 1 (2 pg) and 0.1 (0.2 pg) cell, the reproducibility starts to decrease. From this experiment, we concluded that variation in antigen expression on the level of one cell per sample could, indeed, explain the lack of reproducible quadruplicate testing. Ten cells (20 pg/2 μg) were detected in all four cases, whereas one cell (2 pg/2 μg) was detected in one of four or two of four cases. In fact, even on the level of 0.1 cell per 7 ml of blood (0.2 pg/2 μg), positive samples were detected in both cases, suggesting that the assay is sensitive beyond the level of one cell per 7 ml of blood. Nevertheless, when using quadruplicate assays (one reverse transcription, four PCRs), we could detect one single tumor cell reproducibly in all cases (at least one of four PCR positive). We, therefore, decided to use quadruplicate assays for the screening of patient samples. Moreover, this observation enables the use of this dilution (2 pg/2 μg or 5 pg/5 μg) as external quality control of every RT-PCR assay. Even slight changes in sensitivity will be measurable, making the assay very well controlled. Intriguingly, when using quadruplicate assays, we can use the lack of reproducibility within the assay as an estimate of the number of tumor cells present in the clinical specimen, provided that the antigen expression of the tumor is known.

**E48 RT-PCR on Blood and Bone Marrow Samples of HNSCC Patients and Controls.** The optimized RT-PCR test with external control was used on blood and bone marrow samples of HNSCC patients (twice in duplicate) and controls (in quadruplicate). A result was scored as positive when only one of the PCRs was positive. A typical result is depicted in Fig. 6. Besides the results of a subset of the patient material, the serial dilution of RNA isolated from HNSCC cell line UM-SCC-22A in WBC RNA corresponding to 1 or 10 cells per 7 ml of blood (5 pg/5 μg and 50 pg/5 μg), which is included routinely in parallel as external control, is also indicated. The patient characteristics and the test results are summarized in Table 1. The bone marrow RNA and the blood RNA fulfilled the quality requirements (positive Abelson RT-PCR) for 23 and 21 of 28 patients, respectively. The results are summarized in Table 2: 4 of 11 bone marrow samples of HNSCC patients with stage IV disease were positive for E48 RT-PCR, and 4 of 6 samples from patients with recurrent disease were positive. Blood samples taken just before surgery were positive in one of two HNSCC patients with stage II disease and one of nine...
HNSCC patients with stage IV disease. Remarkably, in blood samples taken during surgery (after tumor resection and before reconstruction), positive signal could not be detected. Furthermore, in total, 29 bone marrow and 29 blood samples of noncancer controls were tested, and positive signal was not detected in any of these cases.

Discussion

In the initial study of Schlimok et al. (12), micrometastases of tumor cells were visualized by CK18 immunocytochemistry in the bone marrow of colorectal cancer patients. In larger prognostic studies, a significant correlation between the presence of tumor cells in the bone marrow and disease-free interval and survival was established in patients with colorectal cancer and non-small cell lung cancer (21, 22). Also in head and neck cancer patients, it was shown by immunocytochemical staining with CKs as marker that, in ∼35% of the HNSCC patients, tumor cells were detectable in the bone marrow (23, 24). Interestingly, using E48 RT-PCR, we also found positive signal in 35% of the bone marrow samples.

Immunocytochemical staining is laborious and of limited sensitivity. Furthermore, it is often difficult to decide whether an immunocytochemically stained cell should be considered a cancer cell, and positively stained cells are not necessarily alive. RT-PCR is less laborious, it has a high sensitivity, and the presence of intact RNA indicates that the cells detected are most likely undamaged and viable. During the last decade, a large number of studies using RT-PCR have been reported showing circulating tumor cells in bone marrow and blood. Notwithstanding the perspectives of these sensitive RT-PCR strategies, it has already been shown by several authors that these methods are hampered by illegitimate expression in subsets of bone marrow or blood cells (33, 34), resulting in positive signals in control samples.

A suitable marker for squamous cancer has not been described as yet, and most squamous differentiation antigens are illegitimately expressed in blood and bone marrow as well. On the basis of the molecular characteristics of the E48 antigen, we were able to select a primer set that can be used for squamous tumor cell detection. After a large number of optimization steps, we were able to detect reproducibly a single E48-expressing HNSCC cell per 7 ml of blood (∼2 × 10^7 nucleated cells), but a cumulative testing had to be used: a single 20-ml reverse transcription reaction with four subsequent PCRs. This effect of cumulative testing is of critical importance for a correct evaluation of the data and the proportion of positive reactions in a sample (one of four, two of four, three of four, or four of four) merely reflects the number of tumor cells present in that sample or the relative E48 expression of the detected cells. When an indication of the level of E48 antigen expression is obtained by immunohistochemical staining on frozen biopsies of the tumor, then the number of tumor cells present in the sample can be estimated from the positive results in the quadruplicate assays. In a comparable study Soeth et al. (35)
have used CK20 PCR to detect circulating gastrointestinal tumor cells, and they used duplicate analyses. In difficult cases, when one reaction was positive and the other was negative, a third reaction was performed. When this repeated assay was negative, the sample was presumed to be negative, and when it was positive, the sample was presumed to be positive. When they had used a cumulative testing, as we described, then all these samples should have been considered positive. It is obvious that the analysis of a large number of samples from noncancer controls and the introduction of external controls on the (mimicked) level of 1 and 10 cells per sample are necessary to notice these effects.

Analysis of blood and bone marrow samples of patients with a squamous carcinoma of the head and neck revealed that, in 8 of 23 patients, positive signal in the bone marrow was found. In fact, we found 10 of 25 positive samples, but 2 were excluded because the Abelson control PCR was negative. It should be noted that, in these pilot experiments, we had to exclude samples based on a negative Abelson PCR. There are two main reasons for this problem. A major problem was the processing of the material directly after aspiration. By changing the logistic organization in the clinic, we lowered the number of negative Abelson controls to below 5%. Nevertheless, it did not drop to zero, and we cannot exclude that the absence of Abelson signal in these cases is related to HNSCC patient-related factors. There are two arguments for this statement. (a) Only HNSCC patient samples were, in a few cases, Abelson negative, whereas in the control samples, Abelson failures had not been observed. (b) We observed positive E48 signal in two bone marrow samples, whereas the Abelson control was negative. These observations suggest that either the Abelson transcript level is down-regulated in the lymphocyte population of these patients or the quality of the RNA isolated from the lymphocytes is poor, in contrast to the quality of the RNA isolated from the tumor cells that were present in the bone marrow. These observations might well be related to differences in the immunological constitution of patients versus controls because head and neck cancer patients often suffer from severe immunosuppression. Currently, we are testing other control primer sets on these samples. Obviously, these observations clearly demonstrate the importance of adding appropriate quality controls.

Remarkably, positive signal was not found in blood samples taken during surgery: after tumor resection and before reconstruction. It has been shown earlier that a large number of prostatic tumor cells enter the circulation during surgery and that the presence of a persisting population appeared to be a high risk factor for the development of recurrent disease (20). These results could be explained by the different nature of the disease. Prostatic cancer has the proclivity to metastasize hematogenically, whereas HNSCC is thought to metastasize initially via the lymph, as judged from the clinical disease progression. It should be noted, however, that the marker used, prostate-specific membrane antigen, is illegitimately expressed in ~50% of the bone marrow samples of normal controls, which might have severely influenced the observed results in blood of prostatic cancer patients (20, 33). Finally, one should keep in mind that the blood samples were taken from the arterial line and, thus, after passage of the lung capillary bed. Although it seems obvious to expect circulating tumor cells during surgery, the negative results might be explained by the sampling method used, and the jugular vein might be more suitable for sampling than the arterial lines.

Notwithstanding the positive signal in 35% of the bone marrow samples of HNSCC patients, we can conclude, based on the low number of tumor cells present in the bone marrow and the lack of cells in the blood, that the number of HNSCC tumor cells that spread through the blood is relatively low. When the external controls run in parallel, which correspond to 1 and 10 cells per 7 ml of blood, are compared to the fraction of positive signal in cumulative testing of the clinical material (see also Fig. 6), we might conclude that only a few positive cells are found, which is in agreement with the results in HNSCC and other tumor types (13, 14, 22–24). The cells that are released from the tumor circulate are most likely trapped in the bone marrow, which would explain the relatively high frequency in bone marrow samples as compared to peripheral blood.

The most important question that remains is whether these cells are of clinical importance. On the one hand, they could be apoptotic and not play a role in the progression of the disease. This hypothesis might be true for immunohistochemically stained cells as proteins expressed by normal squamous cells are stable and remain detectable even on exfoliated cells. However, the presence of squamous RNA suggests at least an intact membrane, indicating that these cells are alive. The clinical impact of these cells is currently being determined in a large follow-up study. It seems reasonable to hypothesize that the presence of these cells are a high risk factor for the development of distant metastasis. It might well be that, when the diagnostic procedures and therapeutic interventions increas-

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<th>Noncancer controls Before surgery</th>
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<td></td>
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<td>Total</td>
<td>8/23 (35%)</td>
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*Values represent no. of positive samples/total no. of samples. BM, bone marrow; Bl, blood.
ingly improve the locoregional control, the frequency of clinically manifest distant metastasis will be the price of success. A test to select these patients at a high risk for distant metastasis might be of great value for further therapeutic studies. At this moment, a Phase I clinical trial with 186Re-labeled chimeric anti-CD44v6 antibody has started at our department, and this test might be well suited to prevent over- and undertreatment of patients with residual disease. Combination with the resection margin analysis using p53 mutations as tumor marker, as described by Brennan et al. (11), might really improve the selection of patients at extreme risk of developing recurrent disease, either locoregional or at distant sites.

References
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