Histopathological Characteristics in Squamous Cell Carcinoma of the Oral Cavity with
Regard to Presence of Circulating Tumor Cells

A Thesis

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ABSTRACT

Problem: In 2010, 23,880 new cases of oral cavity cancer were diagnosed and these were associated with 5,470 deaths. The prognostic implication of circulating tumor cells (CTCs) in oral squamous cell carcinoma (OSCC) has not been well-defined. The objectives of this research were: 1) To present and discuss a high performance negative depletion methodology for the isolation of CTCs in the blood of patients with OSCC, 2) To investigate whether or not a correlation exists between the presence of CTCs with specific histopathologic criteria and clinical outcomes.

Materials and Methods: A negative depletion process was used to isolate and quantify CTCs from the peripheral blood of patients with OSCC. This was accomplished by using an immunomagnetic separation method that was previously developed and validated. A histopathological review and prospective clinical follow-up study was evaluated from patients who had their peripheral blood examined for the presence CTCs at the time of surgical intervention. This study included 24 patients, 26 to 81 years of age whom were diagnosed with OSCC. Blood samples were collected between March 2007 to July 2009 and an immunomagnetic enrichment method was used to begin the process of identifying circulating tumor cells. Immunostaining for cytokeratin was used to identify circulating CTCs, which were manually counted using fluorescent microscopy. A correlation

analysis was performed to determine whether or not the presence of CTCs correlated with patients' pathologic tumor stage, as well as the presence of perineural invasion, and lymphovascular invasion. In addition, a Kaplan-Meier plot was created to determine the significance of the presence of CTCs with regard to disease-free survival.

Results: For the 24 patients with OSCC, 17 (70.8%) had the presence of CTCs using the described negative depletion enrichment method. To date, pathologic stage and perineural invasion do not appear to correlate with the presence of CTCs, however lymphovascular invasion showed significant correlation ($p=0.04$). Clinical follow-up (range: 5-48 months), showed an early trend toward improved disease-free survival that was seen in patients with no identifiable CTCs, however, with longer follow-up, this has not shown significance ($p=0.34$).

Conclusions: An enrichment technology, based on the selective removal of normal cells, was used on the peripheral blood of patients with OSCC to identify CTCs. Histopathological characteristics of perineural invasion and tumor stage, which tend to relate to poor prognosis, do not appear to relate to the presence of CTCs. The presence of CTCs did correlate with lymphovascular invasion. The presence of CTCs was associated with a more aggressive early clinical course, and this brings into consideration the use of peripheral blood screening for CTCs as prognostic tool for patients with OSCC.

Dedicated to

Kris Jatana

The most brilliant, understanding, and loving husband

Thank you for your unconditional support

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Chapter 1: Introduction

Despite diagnostic and therapeutic advances by the introduction of multimodal therapy, the 5-year survival rate of patients with oral squamous cell carcinoma (OSCC) remains poor, often due to recurrence or metastatic disease¹. In 2010, per the National Cancer Institute, 23,880 new cases of oral cavity cancer were estimated to occur in the United States with approximately 5,470 deaths attributed to these cases. It is accepted that greater than 95% of these cases are squamous cell carcinomas. With all stages combined, the 5-year survival rate is approximately 50% and this rate has not changed significantly in the past 3 decades.²⁻⁴ Failure in treatment of patients with squamous cell carcinoma of the oral cavity (OCSCC) can include local recurrence, regional recurrence (cervical lymph nodes), distant metastasis, or development of a second primary cancer. Even with therapeutic treatment of the primary lesion and with surgical resection including negative histopathological margins, approximately 50-60% of these patients will later develop locoregional recurrence, and 20% developing distant metastasis. It is possible that the lack of improvement in patient outcomes may be due to early dissemination of cancer cells which are not incorporated into current staging modalities.⁵⁻⁹ Between 23-27% of patients with OCSCC with a clinically negative neck will have micrometastatic disease present in cervical lymph nodes removed during elective neck dissection.^{10,11} Having regional lymphatic metastasis, which is considered advanced stage (3 or 4), is currently the most significant prognostic factor affecting survival and the presence of this can reduce survival by 50%.^{12,13} These current statistics show an important need for a reliable blood marker to determine prognosis in patients with OSCC, specifically those who may be at higher risk of locoregional or distant recurrence.¹⁴

In breast cancer research, it is believed that the process of metastasis occurs early in tumor development.^{15,16} Cancer metastasis may develop when cells from the primary tumor become locally invasive entering lymphatic or vascular channels. Detection of such cells, known as circulating tumor cells (CTCs) in patients with newly diagnosed solid tumors could be a prognostic indicator of future metastasis.¹⁷ The detection of the presence of tumor cells outside the primary tumor could serve 3 purposes: 1) as evidence for early occult spread of tumor cells; 2) as a risk factor for future metastasis and in such giving poor prognosis; and 3) as a marker for monitoring treatment susceptibility; and 4) cancer surveillance.

To date, there are several different types of isolation methods used to find cancer cells in peripheral blood samples: 1.) flow cytometry; 2.) immunocytochemistry; and 3.) reverse transcriptase-polymerase chain reaction (RT-PCR).¹⁸ The first, flow cytometry, the tumor cells are identified by size, granularity, and fluorescent signals that are emitted by fluorescently labeled antibodies that target cell surface markers. There are reagents, such as EpCam and Her-2/neu, that available commercially to target surface antigens common to many CTCs.¹⁹⁻²² In specific fixation procedures, certain intracellular markers such as cytokeratins, also can be stained. Flow cytometry is known to be time consuming when once the number of cells to be evaluated are larger than 10^6 cells. A known alternative would be to sort the cells before using a magnetic enrichment technology and analyze cells with a flow cytometer²³.

The second mentioned, immunocytochemistry, the cells are visually identified by specific morphology and as well as ligand-specific monoclonal antibodies that are conjugated

with fluorescent or brightfield dyes. Intracellular or surface markers are used as target antigens. Although the advantage to using immunocytochemistry is due to the fact the tumor cells are observed directly under a microscope, the disadvantage is that it is quite difficult to find tumor vs. non-tumor cells at a frequency of $1/10^5$ and lower.²³

The third mentioned, RT-PCR, detects tumor cells by targeting specific mRNA which would typically only be present in cancer cells. Such markers include: cytokeratin 8, 19, carcinoembryonic antigen (CEA), and epidermal growth factor receptor (EGFR). RT-PCR studies make the assumption that if the mRNA is present then a CTC is also present. There is no visual cellular confirmation, however, with this assumption. The results tend to be more qualitative than quantitative, however, giving poor specificity.^{22,24}

Explanation for the difference in performance is that expression of the tumor-specific genes in a normal cell may not be zero, but near zero. Thus, a number of normal cells expressing this tumor specific marker, even at a lower level, could make a sample appear as if containing a tumor cell (false-positive).²⁵ This has been called the illegitimate expression of a gene and has been previously described for CK19.²⁶

Tong et al. began attempts to quantify the sensitivity of RT-PCR for CTC markers in human blood spiked by head and neck squamous cell carcinoma cell lines by quantifying the number of cells and the amount of mRNA for EGFR needed for a positive detection. It is known that EGFR is a potential marker for CTC of human oral squamous cell carcinoma. However, it is difficult to positively identify mRNA for EGFR in spiked blood samples, as the working concentration of a cancer cell in blood ranges from 1 in 1,000 to 1 in 100,000 cells. Essentially attempting to find CTC in peripheral blood is like finding a “needle in a haystack”.²⁷ To understand this from a quantitative perspective, Table 1, lists the number of erythrocytes, leukocytes, and platelets per mL of normal

blood. Given the fact erythrocytes constitute approximately 30-40% of the blood volume, the complete removal, without any loss of other cell types, is needed for accurate CTC detection. In order to make CTC detection routine, technology needs to be developed which will enrich the CTCs from the blood in a simple and inexpensive way. The goal would be to remove all the normal cells leaving only the CTCs behind, however, this methodology would require an approximate 7-8 $\log_{10}$ enrichment.²⁵

Type of cell	Average number of cells/per mL of fresh blood
Erythrocyte (RBC)	5,000,000,000 (range from 4.4 to 5.9 x 10 ⁹)
Platelet	295,000,000 (range from 150 to 440 x 10 ⁶)
Leukocytes (WBC)	7,000,000 (range from 3.9 to 10.6 x 10 ⁶)
Leukocyte Compositions	
Lymphocytes	2,400,000 (range of 1.2-3.5 x 10 ⁶)
Monocytes	300,000 (range of 0-0.5 x 10 ⁶)
Granulocytes	4,000,000 (range of 1.4-6.6 x 10 ⁶)

Table 1: Typical Composition of Human Blood²⁸

A negative depletion method would involve the removal of normal cells, such as RBCs through lysis and lymphocytes through magnetic depletion of CD45⁺- labeled cells. This is in contrast to a positive selection method where selection of tumor cells are performed by magnetic selection of epithelial cells using antibodies binding to surface markers such

as epithelial cell adhesion molecule (EpCAM). Prior published papers used a combination of both for detection of CTCs. One step involved removal of RBCs (negative depletion) and the second was a positive selection of CTCs using the antiepithelial antibody bound to a magnetic particle (as described above). The CellSearch System by Veridex LLC, Raritan, New Jersey is the only FDA approved, commercially available system, and it uses EpCAM for CTC identification.²⁹⁻³¹

In positive selection, CTCs must express the surface marker to which the antibodies are specific, allowing for a potential bias. Sieuwerts et al demonstrated this bias by experiment, specifically showing the CellSearch System effectiveness varied with its ability to detect different subtypes of human breast cancer cells spiked into healthy human blood. From prior studies, it is known the CellSearch System was able to recover 85% of the breast cancer cells displaying normal epithelial characteristics. Sieuwerts et al found that as the cell types expressed a different phenotype characteristic of tumor-initiating stem cells, the recovery of the spiked cells dropped to only 2%. They noted that EpCAM may be down regulated in epithelial-to-mesenchymal transitions (EMT) giving the cancer cells a more invasive phenotype. Due to EMT, using positive selection technique may not allow for proper identification of CTCs in some patients.^{25,32}

Due to the fact that using a positive selection technique may not identify all CTCs, Yang et al. developed an enrichment method that is based only on negative depletion of normal cells.²⁵ These researchers were able to optimize this method to obtain an average of 5.66 log₁₀ enrichment of CTCs in the blood of patients with head and neck cancer.¹⁴

Chapter 2: Materials and Methods

Material and methods were optimized by Yang et al.¹⁶ and Tong et al.²⁷ and will be summarized below. The multi-step procedure included collection of blood samples, cell concentration analysis, red cell lysis, negative separation of the normal blood cells from the CTCs which is done in preparation for the final step; immunocytochemistry analysis.

1. Blood Collection

The collection of blood specimens from patients undergoing surgery for OSCC was approved by the institutional review board and study patients were given informed consent at the James Cancer Hospital and Solove Research Institute at The Ohio State University, Columbus, OH. Blood samples of 10 to 18 mL were drawn at the time of surgery. The blood samples were collected in green-top BD Vacutainer tubes (BD Vacutainer Systems, Franklin Lakes, New Jersey) and then were stored at 4°C until processing, which occurred within 24 hours after collection. The researchers were blinded to all clinical and histopathological correlative data during the sampling and analysis process. The blood samples from buffy coats spiked with the appropriate number of cancer cells from cell cultures.

2. Overall Enrichment Process

Cell concentration determination was done by taking the fresh blood from the green top Vacutainer tubes and diluting the sample in a labeling buffer. The final number was

determined by the Improved Neubauer Hemocytometer. To obtain the concentration of peripheral blood lymphocytes and PBLs in the samples before red cell lysis, after red cell lysis, and after magnetic cell separation, an aliquot of the sample was diluted in 3% acetic acid (1:25, 1:25, 1:10 dilutions) in a 1.5 mL Eppendorf tube and left to stand for 10 minutes at room temperature to lyse all red blood cells. When 10 minutes of incubation had passed, the sample was vortexed to prevent induction of bubbles. A cover slip was put into place and a drop (10-20 μ L) of the well-mixed sample was placed in the notch and the sample was left to fill the chamber by capillary action. This was left undisturbed for 1-2 minutes to allow cells to deposit on the counting plane. The number of PBLs was next determined and concentration reported. Diagram of the overall enrichment process is shown in Figure 1.

Figure 1: Overall Enrichment Process²⁵

3. Red Cell Lysis

The red cell lysis step began with incubating a ratio of 25 mL lysis buffer to 1 mL of blood at room temperature for 5 minutes. The sample was centrifuged at 300g, washed and then re-suspended in labeling buffer (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin). The concentration of nucleated cells was determined by using the Unopette® Microcollection system hemacytometer (Cat # 365856, BD Biosciences, San Jose, CA). This suspension of nucleated cells was ready for magnetic labeling.

4. Negative Separation Methodology

The cell suspension obtained after cell lysis, consisting mainly of nucleated cells, was first labeled with FcR blocking agent and then anti-CD45 TAC and incubated for 30 minutes at room temperature on a shaker. Without washing the cells, the magnetic nanoparticles were then added to the cell suspension and incubated for 15 minutes at room temperature in a shaker. The immunomagnetically labeled cell suspension was subsequently run through the deposition Quadrupole Magnetic Sorter (dQMS) System to obtain an enriched sample containing CTCs. The enriched sample containing the suspended CTCs was then divided into multiple aliquots; one of these was subjected to a cytopspin for immunocytochemistry staining, and a second aliquot was lysed to obtain RNA for further molecular analysis.

5. Immunocytochemistry Analysis

The staining of tumor cells began by placing the enriched suspension after magnetic

separation into 2 different aliquots: 1.) immunocytochemistry (ICC); and 2.) RT-PCR. For this study, ICC alone was used for our results and is summarized. For ICC, the cytospin was obtained using a Shannon cytospin instrument (Thermo Scientific, Pittsburgh, Pennsylvania) and fixed in 1% formaldehyde for 10 minutes. For staining, a 1:10 dilution of the anticytokeratin fluorescein isothiocyanate conjugated (FITC) antibody CK306H5 (MiltenyiBiotec, Auburn, CA) recognizes cytokeratins 8, 18, and 19 was then applied to the cytospin and incubated at 37°C for 30 minutes in a humidity chamber. Each slide was then washed in PBS 3 times for 5 minutes each and then allowed to air dry. These slides were then mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California). Slides were observed on a Nikon fluorescent microscope (Nikon Corp, Tokyo, Japan), equipped with filter for DAPI and FITC emissions. Per Partridge et al. and Reithdorf et al. in order to identify a CTC, the cell must have: 1) be double positive for FITC and DAPI staining; 2) have an intact membrane; 3) have a high nuclear to cytoplasmic ratio and be large, and 4) be negative for CD45-PE. Due to our protocol, criteria # 4 was indirect as we magnetically labeled the CD45-positive lymphocytes and magnetically removed them, therefore the remaining cells were not CD45 positive. The final concentration of CTCs per mL of peripheral blood was calculated using the number of CTCs on the cytospin using appropriate dilution factors during the negative depletion process. Figure 2 shows a representative photograph of this technique.

Figure: 2. Circulating tumor cell (CTC) immunocytochemistry technique. A. Light microscopy; B. cytokeratin--cytoplasm stains green); C. DAPI (4, 6-diamidino-2-phenylindole)--nucleus stains blue; and D. combined cytokeratin and DAPI with high nuclear to cytoplasmic ratio (defined a CTC); (original magnification x 200 for all images).²⁵

In light of the rigorous multi-step methodology the following inclusion criteria were applied to the clinical data: 1) final pathological diagnosis of OSCC; 2) successful enrichment (after RBC lysis, depleting nucleated cells by at least 1 log₁₀ enrichment); and 3) that clinical follow-up data exists. Unless a high level of enrichment was achieved, the ability to detect CTCs in peripheral blood can be questionable.

6. Statistical Analysis

A Kaplan-Meier Survival Curve in this study is used to plot the probability of disease free survival (y-axis) longitudinally over time (x-axis), which is a function of months after the initial sample was taken for 2 groups: A) CTCs present, and B) No CTCs present. At time zero, by definition, all patients would be alive with no cancer recurrence ($y = 100\%$). As time progress and patients are found to have disease recurrence and/or cancer-related mortality, the percentage decreases. For each time interval, the disease free survival probability, is calculated as the number surviving over the number at risk at the end of each time interval. An analysis based on current disease-free survival data using an 80% probability of detecting a difference at the 5.0% significance ($p=0.05$) level was carried out to determine the number of patients needed for future clinical studies.^{33,34}

A statistical analysis using the chi-square test (χ^2) and t-test ($\alpha= 0.05$) were used to test the significance between the two groups of patients with regard to several variables found in Table 2. Specifically this tests the null hypothesis: there is no difference between group A (CTCs present) and group B (no CTCs present), with a corresponding alternative hypothesis: there is a difference between group A (CTCs present) and group B (no CTCs present). To compare groups A and B, the chi-square test (χ^2) was used for analysis of variables that are not normally distributed where as the t-test was used for variables where the data is normally distributed. Statistical analysis was performed with the log-rank test for Kaplan-Meier survival plot (Figure 4) and chi square-test and t tests for calculation of p-values (Table 2).

Chapter 3: Results

Applying the inclusion criteria (final pathological diagnosis, successful enrichment, and knowledgeable clinical follow-up data), 24 patients were identified. The mean prospective time of follow-up for patients not presenting with a recurrence or cancer-related mortality was 28.1 months (SD=13.0). CTCs were identified in 17 patients (70.8%) and while no CTCs were present in 7 patients (29.2%) as shown in Figure 3. The Kaplan-Meier as shown in Figure 4 was created. This shows the probability of disease free survival over time with regard to the presence of CTCs. While the early presence of CTCs corresponded to disease recurrence, this did not maintain statistical significance in the long term ($p=0.34$).

Figure 3: Distribution of circulating tumor cells (CTCs) per millimeter in peripheral blood of patients with OSCC: bar on left, 7 patients (29.2%) had no CTCs; bar on right, 17 patients (70.8%) had CTCs present.

Clinically detectable cancer recurrence was as early as 4 months and as late as 37 months after the initial blood samples were drawn. A summary of patient characteristics including age, sex, overall stage, nodal status, adjuvant therapy, smoking, alcohol use, histopathological characteristics is provided in Table 2. Overall, there were no significant differences between these 2 groups with regard to these variables, except for lymph vascular invasion ($p=0.04$).

Characteristic	CTCs Present n = 17	No CTCs Present n = 7	p-value
Age, range, y	62.0	50.4	0.15
Men %	52.9	62.5	0.42
Women %	47.4	28.6	0.42
Smoking > 15 PPY No. (%)	73.7	42.9	0.11
EtOH, Moderate, No. (%)	47.3	28.6	0.30
Follow-up, mean, mo	25.6	33.4	0.24
Overall Stage, No. (%)			0.84
1/2	47.1	57.1	
3/4	52.9	42.9	
Pathological node, No. (%)	21.1	42.9	0.32
Perineural Invasion, No. (%)	57.9	42.9	0.83
Lymphovascular Invasion, No. (%)	23.5	0.0	0.04
Tumor Differentiation			
Well	52.9	57.1	0.86
Moderate	41.2	28.6	0.58
Poor	5.9	14.3	0.60
Adjuvant therapy, No. (%)	57.9	42.9	0.94
Total cell log depletion	5.38	5.57	0.58

Table 2: Patient Characteristics Including Age, Sex, Stage, Nodal Status, Adjuvant Therapy, Smoking, and Alcohol Use. Abbreviations: CTCs (circulating tumor cells), EtOH (ethanol), and PPY (packs per year)

Figure 4: Kaplan-Meier disease free-survival plot with regard to the presence of absence of circulating tumor cells (CTCs) in peripheral blood. P-value=0.34.

Based on the current disease-free survival data, as shown in Figure 4, the median time to cancer recurrence was 5.5 months in the CTC group and the assumed hazard ratio was 0.56. With a planned accrual period of 36 months and a follow-up period of 48 months, a total of 73 patients would be required.

Chapter 4: Discussion

CTC detection in patients with OSCC is relatively uncharted field of study when compared with other cancers, such as breast, prostate, colorectal, and neuroblastoma. Despite improvements in surgery and reconstructive techniques, 20-30% of patients presenting with early tumors and more than 50% of those with advanced OSCC, fail to survive 5 years post-treatment.³⁵

In this study, by using a negative depletion methodology, an average of 5.66 log₁₀ total enrichment of CTCs in the blood of patients with OSCC was found. Such a method leaves CTCs in their native state to allow further unaltered characterization. The red blood cells are removed by lysis, followed by a magnetic separation of immunomagnetically labeled CD-45 positive leukocytes, which then leaves the potential CTCs for further characterization. Immunocytochemical staining was then performed using nuclear staining and cytokeratin. This study showed that by using this high-performance negative depletion technique, early detection of CTCs in the peripheral blood of patients with OSCC were visually confirmed and predictive of disease recurrence, however, this did not hold true in the long-term. The absence of CTCs may be promising as an early indicator for disease survival but continued prospective follow-up on these patient outcomes is needed. Further characterization of these samples with multi-marker analysis including mesenchymal and cancer stem cell markers may identify sub-groups of patients at risk for cancer recurrence. Sample size is certainly a limitation of this study and with the addition of more patients, further analysis of the variables assessed may ultimately show statistical significance. Confirmation of the reliability of the cell identification also is a limitation of this study and requires attention in future studies.

There is evidence that CTCs may be predictive of prognosis in patients with squamous cell carcinoma of the head and neck (SCCHN). Partridge et al. used a negative depletion methodology to identify disseminated tumor cells in SCCHN patients and correlated this with outcomes. They found that the detection of disseminated tumor cells preoperatively or intraoperatively indicated an increased risk of local/distant recurrence and reduced survival. Jatana et al.¹⁴ published that 48 patients with SCCHN of multiple sites (oral cavity, oropharynx, hypopharynx, larynx), with a mean follow-up of 19 months demonstrated a statistically significant worse disease-free survival in patients with CTCs present at the time of surgical resection.

There is very limited literature available for OSCC alone. It is possible that site specific data with regard to OSCC is important to the ultimate analysis of the data for prognostic implications. In 2009, Toyoshima et al. evaluated cytokeratins 17, 18, 19, and 20 with RT-PCR on 40 patients 4 weeks postoperatively of OSCC surgical resection. They did not detect CK-17 and -19 in any samples, but were able to statistically show ($p=0.04$) that CK-20 was higher in patients with T3 and T4 OSCC. The authors felt CK-20 in peripheral blood could play relevance for prognosis of patients with OSCC¹. In our study, we were able to show correlation of lymphovascular spread to the presence of CTCs. We were also able to confirm that there is a group of patients with *early presence of CTCs* had a worse disease-free survival on with the Kaplan-Meier estimated disease free survival plot. This finding could help direct more early aggressive treatment therapy for patients with OSCC and CTCs. Other histopathological prognostic findings such a perineural and lymph node involvement did not show a statistical difference. It is possible that the presence of CTCs may be an *independent* prognostic factor in OSCC. Future studies with the addition of 49 patients based on current disease-free survival analysis

will help to further delineate for a statistically significant difference between the 2 groups of patients. Also, further genetic analysis of CTCs may help further characterize the cells for risk stratification for early cancer recurrence.

Chapter 5: Conclusion

There are still many unanswered questions regarding the biological basis and role of CTCs in OSCC. With continued accrual of patients and more prospective follow-up, further characterization of CTCs with additional markers, and genetic analysis of CTCs, the ultimate goal will be to incorporate CTC identification into the management of patients with OSCC. Based on current data, an addition of 49 patients would be required to reach statistical significance for disease-free survival. This will ultimately allow for the development of a prognostic blood test to improve the clinical outcome of patients with OSCC.

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