Development of a standardized flow cytometric method to conduct longitudinal analyses of intracellular CD3ζ expression in patients with head and neck cancer

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common neoplasm in the world. The follow-up protocols currently available do not appear to diagnose treatment failures and recurrences early enough to provide the best treatment to improve the survival rates of patients. The identification of biomarkers may aid in diagnosing, monitoring the progression, or predicting treatment outcomes in HNSCC. The present study aimed to evaluate whether cluster of differentiation (CD) 3ζ chain expression may serve as a biomarker for the early detection of recurrent or persistent HNSCC. However, in a longitudinal study, a standardized method that allows consistent data comparisons in an inter-assay manner is critical. The present study reveals a method to monitor expression levels of CD3ζ over multiple time-points using flow cytometry. The present study validated the use of an internal control and normalization procedure for tracking alterations in CD3ζ expression in samples from patients with HNSCC, which were collected and assayed for a longitudinal study.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is located in the upper aerodigestive tract. Although it is the sixth most common neoplasm in the world, advances in multi-modality treatments, including surgery, radiation and chemotherapy, have not increased the 5-year survival rate of ~50% over the past 35 years (1,2). Identifying biomarkers in the blood or other body fluids of an individual may aid in the diagnosis of disease and predict treatment outcomes in patients with HNSCC (3,4). In a cross-sectional study, analysis of a candidate biomarker uses single time-point sampling from population data (5). By contrast, a longitudinal analysis involves using multiple samples from a single patient at various time points; therefore, this type of analysis reveals quantitative alterations in the candidate biomarker in the patient, which may be associated with improved health, treatment outcomes and clinical status of the patient (6). However, to quantify a candidate biomarker in a longitudinal study, a standardized assay that supports robust and consistent inter-assay data collection is critical for statistical analyses (7).

Flow cytometry is a laser-based technology for the analysis of physical and chemical properties of cells or cellular components. Advances in flow cytometer designs and novel fluorochromes for the chemical conjugation of antibodies have facilitated robust, multi-parametric analysis at a single cell level. The technology has been widely used in basic research (8), clinical analysis of patient immune responses (9), hematological malignancies and minimal residue disease (10-12), and enumeration of lymphocyte populations in immunodeficient diseases and cancer (13,14).

The present study aimed to evaluate whether the expression of cluster of differentiation (CD) 3ζ in peripheral T cells from patients with HNSCC may serve as a biomarker for the early detection of recurrent or persistent HNSCC in a longitudinal study. The present study used commercially available anti-CD3ζ antibodies in flow cytometry to determine CD3ζ expression in a cohort population of patients with HNSCC prior to and following clinical treatments. However, while comparing the longitudinal data obtained over multiple time points, the present study observed that numerous ranges of CD3ζ expression were exhibited in the flow cytometric assays. The present study developed and described a method that conducts longitudinal flow cytometric analysis of a candidate biomarker, such as CD3ζ, in patients with HNSCC.

Materials and methods

Cohort of patients with HNSCC. In total, 24 patients with HNSCC, which consisted of 15 male and 9 female patients,

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were evaluated in the current study. The age range of the patients was 21-94 years, with a median age of 64 years. Histological analysis revealed that 95.83% of the patients possessed cancers of a squamous origin. In total, 16 patients were treated using a single treatment modality, including surgery, radiation or chemotherapy, and 8 patients were treated using a combination of various treatment modalities, including surgery with radiation or chemotherapy (Table I). Peripheral blood (10-15 ml) was collected from the 24 patients and also from 24 healthy control donors. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation, as previously described (15,16). The present study was approved by the Research Ethics Board at the University of Manitoba (Winnipeg, MB, Canada).

Antibodies and flow cytometry. Sequential staining was performed to evaluate CD3ζ expression in T cells using flow cytometry. Surface CD3ε, a marker of T cells, was stained using specific fluorescein isothiocyanate (FITC) mouse anti-human CD3 monoclonal antibody (clone, UCHT1; cat no. 55916; dilution, 1:66; BD Biosciences, Franklin Lakes, NJ, USA). Following surface staining, PBMCs were fixed in 1% paraformaldehyde (Sigma-Aldrich, Ontario, Canada)for 10 min at room temperature, followed by two rounds of washing with phosphate-buffered saline (PBS). In total, 2 ml of cell permeabilization buffer (0.1% saponin and 0.05% sodium azide in PBS; Sigma-Aldrich) was added to the PBMCs. The PBMCs were kept on ice for 15 min in the dark, washed once with cell permeabilization buffer and resuspended in 0.2 ml of the cell permeabilization buffer. The expression of CD3ζ was detected intracellularly, using a specific phycoerythrin (PE)-conjugated monoclonal antibody (clone, 2H2D9; ca no. IM3169; dilution, 1:10; Beckman Coulter, Inc., Brea, CA, USA). An immunoglobulin (Ig)-G1 isotype control (eBiosciences, San Diego, CA, USA) was used to distinguish specific CD3ζ binding from non-specific binding during flow cytometry. The optimal amount of antibody was determined by titration and the cells were analyzed by BD FACSCanto™ II flow cytometer (BD Biosciences). A total of 10,000 events were acquired and the expression levels of CD3ζ in T cells (gated on $CD3\varepsilon^+$) were analyzed using FlowJo software, version 9.6.2 (FlowJo, LLC, Ashland, OR, USA) by the mean florescence intensity (MFI).

Freezing and thawing of PBMCs. PBMCs were isolated from the whole blood of healthy donors and HNSCC patients using Ficoll gradient centrifugation, as previously described (15,16), and washed once in complete medium, consisting of RPMI-1640, penicillin/streptomycin/L-Glutamine and 10% fetal bovine serum (Thermofisher Scientific, Inc., Ontario, Canada). Viable cell numbers were determined by a B4005 hemocytometer and trypan blue viable cell exclusion dye (VWR International, Ontario, Canada) (17). PBMCs were cryopreserved at -196˚C in aliquots in complete medium, which contained a final concentration of 10% dimethyl sulfoxide, using a Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The cryopreserved PBMC was removed from liquid nitrogen, and rapidly thawed in a 37˚C water bath prior to the cells being transferred to 10 ml pre-warm complete media. The cell pellet was collected and resuspended in 1 ml of fresh, warm complete media at 37˚C. The viable cell count was determined, and the thawed cells were used directly for flow cytometric assays.

Table I. Clinical pathological characteristics of 24 patients with head and neck squamous cell carcinoma.

Characteristics	n
Total	24
Gender	
Male	15
Female	9
Age, years	
Median	64
Range	21-94
Histological type	
Squamous cell	23
Merkel cell	1
Treatment	
Surgery	13
Chemo	1
Surgery + chemo + radiation	4
Surgery + radiation	1
Radiation	1
Chemo + radiation	4

Chemo, chemotherapy.

Internal control for flow cytometry. The present study collected PBMCs from 5 healthy donors. The PBMCs of the healthy donors were pooled and stored in small aliquots in liquid nitrogen until use. In each flow cytometric assay, one of these vials was thawed ($0.5x10^6$ cells) and used in the staining process along with the patient samples. To evaluate the intra and inter-assay variation, the same internal control was thawed and the CD3ζ expression was analyzed 3 times using flow cytometry at 3 time points in weekly intervals. The MFI of the CD3ζ chain expression was measured within an individual experiment for intra-assay variability and between multiple experiments for inter-assay variability.

Determination of cell viability and exclusion of dead cells from the analysis. To determine the cell viability and to exclude the dead cells from the analysis, Fixable Viability Dye eFluor® 780 (eBioscience, Inc., San Diego, CA, USA) was used, according to the manufacturer's protocol. The cells were washed with flow buffer (0.05% FBS, 10% sodium azide in PBS) and surface staining was performed using an anti-CD3ε monoclonal antibody. The cells were then kept in the dark at 4˚C for an additional 30 minutes prior to intracellular staining for CD3ζ. Live T cells, gated from the dead cells using either the fixable viability dye or forward scatter/side scatter (FSC/SSC), were analyzed for CD3ζ chain expression.

Data normalization. CD3ζ expression (expressed as the MFI) was determined in the PBMCs of the internal control, healthy

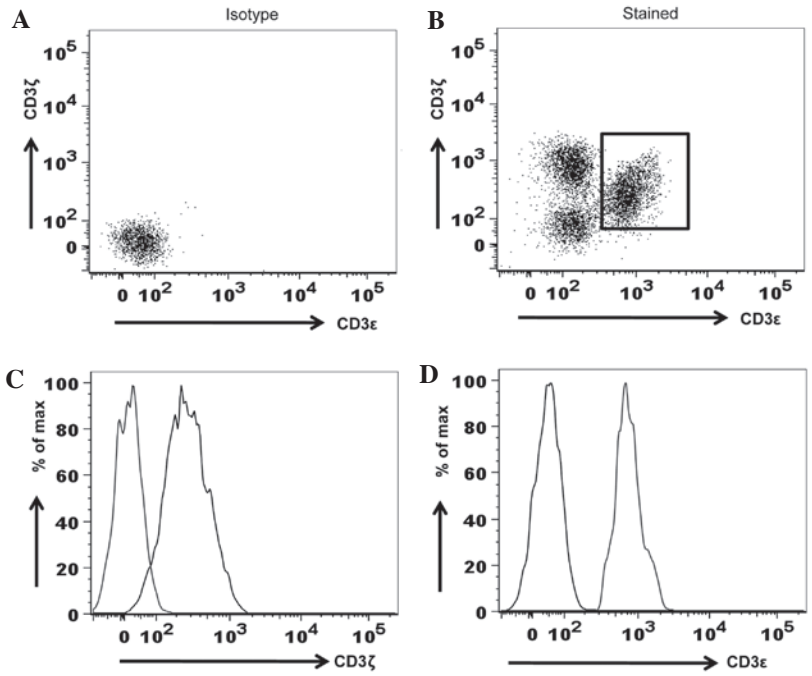

Figure 1. Expression of CD3ζ in CD3$^+$ T cells using flow cytometry. (A) Graphical representation of isotype control staining for CD3ε$^+$/CD3ζ$^+$ cells. (B) Graphical representation of staining of CD3ε$^+$/CD3ζ$^+$ cells. (C) Histograph representing the staining for CD3ζ and the isotype control IgG1. (D) Histograph representing the staining for CD3ε and the isotype control IgG1 κ. Expression of the ζ chain was investigated in 0.5x10^6 cells of quickly thawed peripheral blood mononuclear cells. Surface staining was performed using anti-CD3ε monoclonal antibody followed by intracellular staining using anti-CD3ζ monoclonal antibody in the saponin-permeabilized cells with their respective IgG1, κ and IgG1 isotype controls. The cells were investigated using a BD FACS Canto flow cytometry where 10,000 events were acquired. The CD3ε$^+$/CD3ζ$^+$ cells were analyzed by gating the lymphocyte population using FlowJo software. CD, cluster of differentiation; Ig, immunoblobulin.

controls and patients using an individual flow cytometric assay. Data normalization was performed by transforming the MFI values of the test samples (patients and healthy donor) to a common scale using the following equation: Final relative fluorescence intensity = MFI of the test sample / MFI of the internal control.

Normality and statistical analysis. Normality and statistical analyses of the data were performed using the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), termed the normality test. The D'Agostino-Pearson omnibus test was performed to investigate whether the data passed the normality test. An unpaired *t*-test was applied to the data that passed the normality test. The Mann-Whitney test was used on data that did not pass the normality test.

Results

Flow cytometric analysis of CD3ζ chain expression. To evaluate the specificity of anti-CD3 antibodies in flow cytometry, fluorochrome-conjugated isotype controls, consisting of the PE-mouse IgG1/κ antibody for CD3ζ detection and the FITC-IgG1/κ antibody for CD3ε detection, were used in the surface and intracellular staining of PBMC. The present study demonstrated that,when compared to the isotype controls, anti-CD3ζ and anti-CD3ε antibodies were specific (Fig. 1).

Freezing and thawing of PBMCs may induce cell death and affect the present analysis. Using a commercial fixable viability dye, the present study observed that the percentage of dead cells in the thawed samples were in the range of 2-23% (Fig. 2). To measure CD3ζ chain expression using MFI in live cells, the present study separated live cells from dead cells using the viability dye prior to the analysis of CD3ζ expression in the CD3ε$^+$ T cells. In addition, the present study compared the differences in CD3ζ expression when the live cells were gated by either the viability dye or the FSC/SSC properties of the total cells acquired. The present study observed that there were no significant differences in the MFI determined by these two methods of live cell gating (Fig. 2).

Subsequently, the present study calculated the standard deviation for the inter-assay and intra-assay variations in MFI of CD3ζ expression using various aliquots of the identical PBMC sample thawed and assayed at various time points (weekly intervals for 3 consecutive weeks) for the inter-assay, and triplicate measurements of 1 aliquot of PBMC sample thawed and assayed in a single flow cytometric assay for the intra-assay. The ranges of the variations observed within an assay and between multiple assays were 407-412 and 407-477, respectively. The standard deviations for intra-assay and inter-assay were 2.16 and 28.6, respectively (Fig. 3).

Data analysis with or without normalization. Despite efforts to perform flow cytometric assays under identical conditions, the present study observed larger inter-assay variations in the MFI values of CD3ζ expression during the last 36 months. The present study noted that the MFI values of CD3ζ expression of the identical internal control varied between 378 and 1,106. It

Table II. Raw and normalized data of the MFI of CD3ζ expression in $CD3^+$ T lymphocytes.

	Internal control		Sample	
Run no.	Prior to normalization	Following normalization	Prior to normalization	Following normalization
1	378	1.00	281	0.74
2	1106	1.00	2213	0.71
3	3106	1.00	999	0.90

MFI values of the same PBMC samples (internal control or a test sample) were examined and analyzed in flow cytometry at various time-points. In total, 3 representative experiments are exhibited to illustrate the wide range of variations in the MFI values observed. Using the MFI value of the internal control obtained in each run, the raw data was normalized to represent the relative alterations in the MFI. MFI, mean fluorescence intensity; CD, cluster of differentiation.

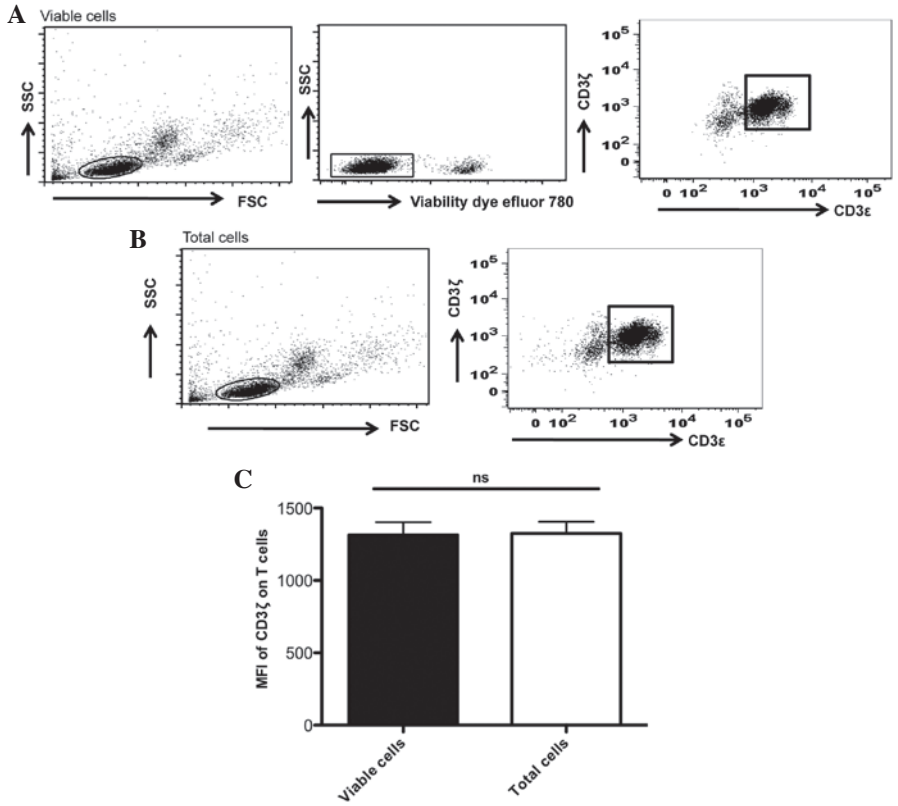

Figure 2. Live-cell gating by either viability dye or forward/scatter properties of the total cells acquired did not affect the MFI analyses of the CD3ζ chain expression. (A) MFI of the CD3ζ chain measured in the viable cells, gated by viability dye. In total, $0.5x10^6$ cells of the thawed peripheral blood mononuclear cells were washed in PBS and resuspended in 200 μl of serum-free PBS. A total of 0.2 μl fixable viability dye was added to the cells, incubated for 30 min at 4˚C prior to subsequent washing and surface and intracellular staining of CD3ε and CD3ζ, respectively. Using BD FACS flow cytometry 10,000 events were acquired. Based on FSC/SSC properties, lymphocyte populations were gated for subsequent discriminations of dead and viable cells by the Fixable Viability Dye eFluor® 780 (eBioscience, Inc.). Only the viable cells were gated in the MFI analyses of the CD3ζ chain in $CD3\varepsilon^+/CD3\zeta^+$ cells. (B) MFI of the CD3ζ chain was determined by live-cell gating using the FSC/SSC properties of the total cells. Live lymphocyte populations were gated by their FSC/SSC properties. MFI of the CD3ζ chain in the $CD3\varepsilon^+/CD3\zeta^+$ T cells were determined. (A and B) These experiments were generated from the same experiment, except the use of the viability dye in (A). (C) No significant difference in the MFI of the CD3ζ expression in the $CD3\varepsilon^+/CD3\zeta^+$ cells was observed when the two methods of live-cell gating were compared. The statistical analysis was performed using Student's *t*-test (GraphPad Prism 5; 5 experiments were performed in total). MFI, mean fluorescence intensity; CD, cluster of differentiation; PBS, phosphate-buffered saline; FSC/SSC, foward scatter/side scatter; ns, non-significant.

was reasoned that an internal control provided not only a quick way to examine MFI variations acquired using various equipment or the same equipment under identical photomultiplier (PMT) voltages, but also a reference value for data normalization in the inter-assays (Table II). Therefore, the present study applied a normalization procedure to the data sets of CD3ζ expression in the PBMC samples of the patients with HNSCC, which represented a low range (MFI, 378; Table III) and a high range of CD3ζ MFI (MFI, 1,106; Table IV). In addition, the present study incorporated a data set collected under an extreme MFI (MFI, 3,106; Fig. 4; Table V) to additionally test the normalization procedure. As revealed in Tables III-V, normalization transformed the data in the low, high and extreme MFI groups into more consistent values.

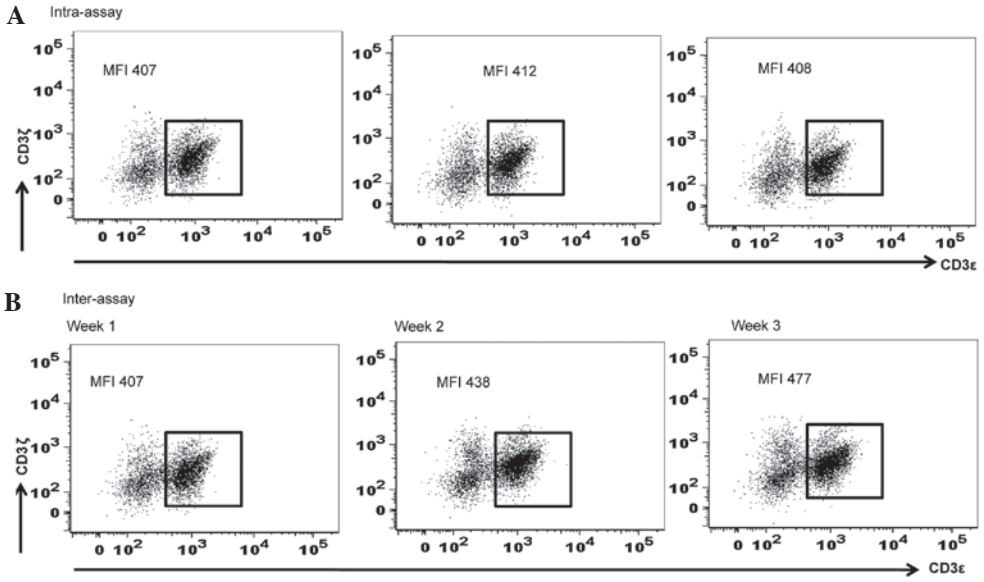

Figure 3. Intra-assay and inter-assay variations in the flow cytometric analyses of CD3ζ expression. Peripheral blood mononuclear cells from normal healthy donors was isolated by Ficoll-Paque density gradient centrifugation and stored in multiple cryovials. (A) Intra-assay analyses, 1 cell vial was thawed and the cells were distributed into a minimum of 3 flow cytometry test tubes ($0.5x10^6$ per tube) for the subsequent surface and intracellular staining of CD3ε and CD3ζ, respectively, and flow cytometric data acquisitions. The standard deviation of CD3ζ chain expression in the intra-assay was 2.16. The intra-assay variation in CD3ζ chain expression was measured by analysing the MFI of the CD3ζ chain in the $CD3ε^+/CD3ζ^+$ cells obtained from the 3 samples performed on the same day. (B) Inter-assay analyses, a cell vial of the same sample was thawed at each time point, in weekly intervals, and the MFI of the CD3ζ expression in $CD3ε^+/CD3ζ^+$ cells was determined and acquired at each time point. Data obtained in these independent flow cytometric experiments were collected and analyzed. The standard deviation of CD3ζ chain expression in the inter-assay was 28.6. CD, cluster of differentiation; MFI, mean fluorescence intensity.

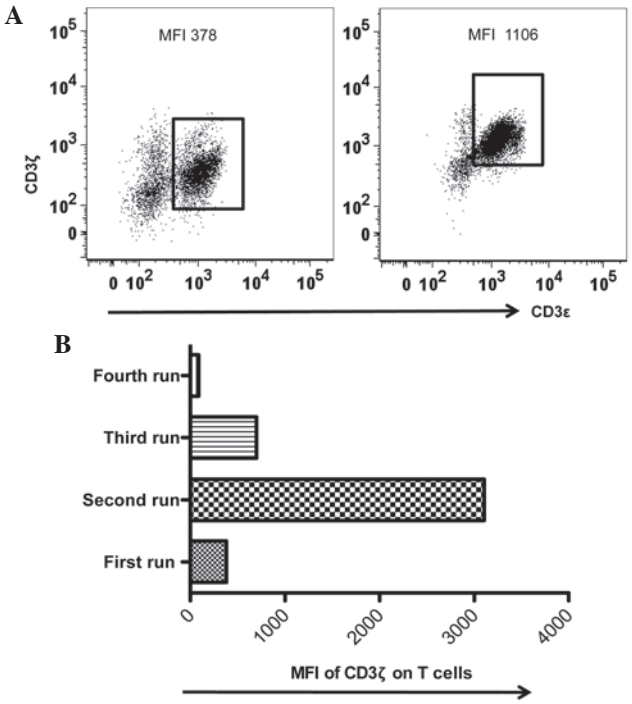

Figure 4. MFI of the CD3ζ varied when acquired at various time points in a long-term longitudinal study. (A) Graphical representation of MFI of ζ in $CD3^+$ T lymphocytes performed under the same PMT voltage at various time intervals. The expression of ζ chain in terms of MFI was analyzed by FlowJo software gated in $CD3ε^+/CD3ζ^+$ cells. (B) An internal control was used in flow cytometry, under the same PMT voltage, at 4 time points. Various MFI values were obtained in these inter-assay analyses. CD, cluster of differentiation; MFI, mean fluorescence intensity, PMT; photomultiplier.

Analysis of CD3ζ expression in healthy controls and patients with HNSCC. Previous small cross-sectional studies have reported a reduced level of the CD3ζ chain in the peripheral blood lymphocytes of patients compared with healthy controls (12,13). Therefore, the present study compared the MFI of CD3ζ expression in the healthy controls and the HNSCC patients prior to and following the normalization process. The present study measured the MFI of CD3ζ

Table III. Data normalization of the MFI of CD3ζ on $CD3^+$ T lymphocytes in a low MFI range prior to and following data normalization.

	Prior to normalization		Following normalization	
Value	Patient	Healthy donor	Patient	Healthy donor
MFI	185	253	0.48	0.66
	248	238	0.65	0.62
	209	256	0.55	0.67
	260	378	0.68	1.00
	282	392	0.74	1.03
	282	340	0.74	0.89
	257	304	0.67	0.80
	212	238	0.56	0.62

The MFI value for the internal control determined using flow cytometric analysis was 378. This value was used to normalize the raw MFI data of healthy donor (n=7) and patients (n=8) samples. MFI, mean fluorescence intensity; CD, cluster of differentiation.

Table IV. Data normalization of the MFI of the CD3ζ on $CD3^+$ T lymphocytes in a high MFI range prior to and following data normalization.

	Prior to normalization		Following normalization	
Value	Patient	Healthy donor	Patient	Healthy donor
MFI	611	1,271	0.55	1.14
	515	1,106	0.46	1.00
	597	1,337	0.53	1.20
	989	675	0.89	0.61
	601	880	0.54	0.79
	614	820	0.55	0.74
	566	583	0.51	0.52
	471	674	0.42	0.60

The MFI value for the internal control determined using flow cytometric analysis was 1,106. This value was used to normalize the raw MFI data of healthy donor (n=7) and patient (n=8) samples. MFI, mean fluorescence intensity; CD, cluster of differentiation.

Table V. Data normalization of the MFI of the CD3ζ on $CD3^+$ T lymphocytes in an extreme MFI range prior to and following data normalization.

	Prior to normalization		Following normalization	
Value	Patient	Healthy donor	Patient	Healthy donor
MFI	2,096	2,758	0.67	0.88
	2,019	2,694	0.65	0.86
	2,356	2,701	0.75	0.86
	2,276	3,364	0.73	1.08
	2,092	3,106	0.67	1.00
	1,972	2,358	0.63	0.75
	2,215	2,108	0.71	0.67
	2,113	2,080	0.68	0.66

The MFI value for internal control obtained using flow cytometric analysis was 3,106. This value was used to normalize the raw MFI data of healthy donor (n=7) and patient (n=8) samples. MFI, mean fluorescence intensity; CD, cluster of differentiation.

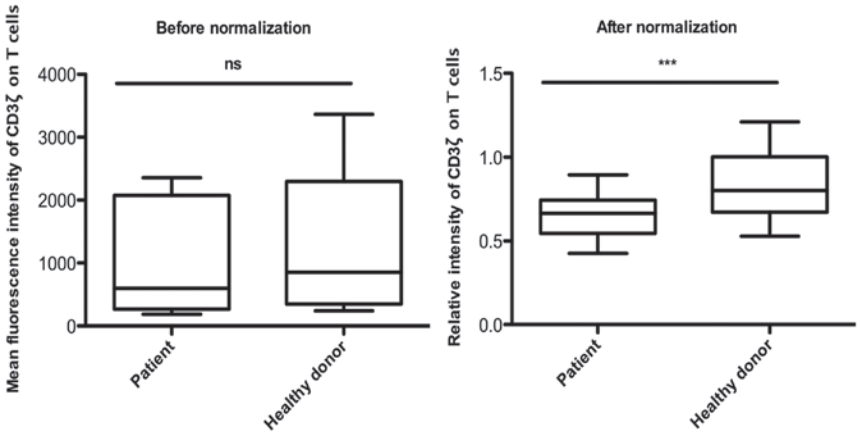

Figure 5. Validation of the data normalization procedure using PBMC samples from the HNSCC patients. MFI of CD3ζ in $CD3^+$ T lymphocytes of healthy and HNSCC patients was analyzed prior to and following normalization. Prior to normalization, there was no statistically significant difference between the CD3ζ expression in the PBMC of the healthy and HNSCC patients. Following normalization, the downregulation of the MFI of the CD3ζ chain on T cells from HNSCC patients was statistically significant compared with that of the healthy donors. ***P=0.0003; n=24. PBMC, peripheral blood mononuclear cell; HNSCC, head and neck squamous cell carcinoma; CD, cluster of differentiation; MFI, mean fluorescence intensity; ns, non-significant.

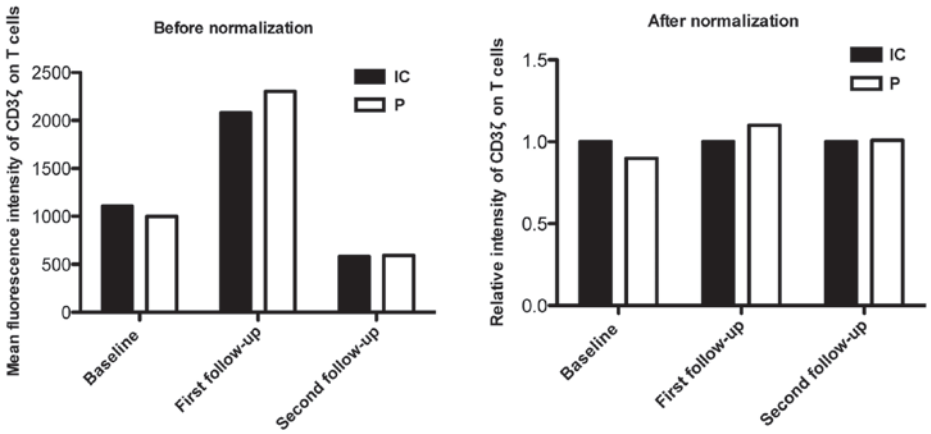

Figure 6. Longitudinal analysis of CD3ζ expression in flow cytometry. P was collected prior to treatment (baseline) and post-treatment (follow-up every 6 months). The expression of CD3ζ was investigated using the standardized flow cytometric method demonstrated in the present study, and was normalized against the IC. Following normalization, the relative CD3ζ expression in the head and neck squamous cell carcinoma sample obtained at multiple time-points were in a closer range. CD, cluster of differentiation; P, peripheral blood mononuclear cells of head and neck squamous cell carcinoma patients; IC, internal control.

expression in the $CD3\varepsilon^+$ T lymphocytes of the healthy control individual and patient samples and normalized the data using the internal control under identical conditions in each experiment. Prior to normalization, no significant difference was observed between the MFI of CD3ζ expression in the healthy controls and the patients with HNSCC. By contrast, following normalization, CD3ζ expression was observed to be significantly decreased in the patients with HNSCC compared with the healthy controls (Fig. 5). Subsequently, the present study validated the normalization procedure in the analyses of CD3ζ expression in a longitudinal study. The present study used longitudinal PBMC samples from a single patient to illustrate the feasibility of using the normalization procedure in a longitudinal study. As revealed in Fig. 6, the use of the internal control during normalization standardized the flow cytometric CD3ζ MFI data acquired at various time points, which in turn allowed each subsequent follow-up sample to be compared to the previous sample, including the baseline data obtained prior to the patient receiving any type of clinical treatment (Fig. 6).

Discussion

The CD3ζ chain is involved in signal transduction and T cell activation (16-18). Growing evidence suggests that ζ-chain degradation in cancer and other malignancies may negatively regulate T cell function (16,19); however, there has not been sufficient evidence to validate the use of ζ-chain expression as a biomarker of disease prognosis. With the wide use of flow cytometry in clinical trials, it is essential to follow gold-standard procedure to quantify biomarkers of disease. The current study developed and validated a method that used an internal control and a normalization procedure to monitor the expression levels of CD3ζ in a HNSCC patient cohort over multiple time-points using flow cytometry.

In a review that summarized a general flow cytometry equipment setup, Maecker *et al* suggested certain setup controls for use in flow cytometric analysis as follows: Gating controls to define positive signals and the use of biological controls, including healthy donors to compare alterations of any factor

associated with alterations in the health of patients (20). The present longitudinal study consisted of an additional internal control for inter-assay variations. PBMC collected from a healthy donor was aliquoted and cryopreserved in multiple aliquots. Each aliquot was used as an internal control; the control was prepared for anti-CD3ζ staining and run alongside other test samples under specific experimental and flow cytometric conditions.

Increasing the PMT voltage is directly proportional to the strength of signals generated from a certain amount of light, and increasing or decreasing the PMT voltage directly affects the output signals and ultimately adds complexity to result analysis (21). Therefore, it is recommended to keep the PMT voltage constant for longitudinal studies. The present study observed that the inter-assay variations assayed in the short term, such as weekly intervals, was reasonably small (Fig. 3). However, despite trying to keep the PMT voltage constant in each flow cytometric analysis, the present study observed that the same internal control produced different MFI values under the same PMT voltage at various time points in a long-term longitudinal study (Fig. 4). These variations may have arisen due to various factors, including a slight alteration in PMT voltage upon each calibration, batch-to-batch variation of the fluorochrome-conjugated anti-CD3ζ antibodies and laser sensitivity. Therefore, a standardized procedure to normalize data obtained in inter-assays was required so that the MFI values were in a consistent range for statistical analyses.

Using a small sample size of healthy control individuals and patients with HNSCC, the present study compared the MFI of CD3ζ expression in the PBMCs of these two groups, with or without applying the normalization procedure. Prior to normalization, statistical analysis indicated that the distribution of data was not normal. There was no statistically significant difference between the CD3ζ expression in the PBMCs of the healthy individuals and patients with HNSCC (Fig. 5). However, following normalization, the present study observed that the distribution of the data became normal. In accordance with previous studies (12,13), the present study identified that the downregulation of the normalized MFI of the CD3ζ chain on T cells was statistically significant compared with the healthy donors (Fig. 5). Transforming the raw data, which had various ranges of MFI, into a normalized scale allowed all the variations in MFI values to become closer in range. This eliminated, not only the effect of inconsistent values generated at multiple cytometric assays, but also enabled a direct comparison of CD3ζ expression data collected at various time-points. Consequently, this method allowed the present study to observe alterations in the expression levels of the molecule of interest using flow cytometry in a longitudinal study (Fig. 6). The standardized method of measuring CD3ζ expression in frozen PBMC using flow cytometry may allow alterations in biomarkers of interest to be tracked by flow cytometry in a large cohort of patients with HNSCC in a longitudinal study.

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