Microarray analyses of oral punch biopsies from acute myeloid leukemia (AML) patients treated with chemotherapy

Jean-Luc C. Mougeot, PhD,a Farah K. Bahrami-Mougeot, PhD,a,b Peter B. Lockhart, DDS,b and Michael T. Brennan, DDS, MHS,b Charlotte, NC CAROLINAS MEDICAL CENTER

Objective. Understanding the pathogenesis of chemotherapy-induced oral mucositis (CIOM) is vital to develop therapies for this common, dose-limiting side effect of cancer treatment. We investigated molecular events in CIOM from buccal mucosa tissue collected before and 2 days after chemotherapy from patients with acute myeloid leukemia (AML) and healthy controls by microarray analysis.

Methods. Microarray analysis was performed using Human Genome U133 Plus 2.0 Array on buccal mucosa punch biopsies from patients with AML before (n = 4) or after chemotherapy (n = 4), and from healthy controls (n = 3). Following Robust Multichip Average (RMA) normalization, we applied Linear Models for Microarray data (LIMMA) and Significance Analysis of Microarrays (SAM) for data analysis using the TM4/TMeV v4.5.1 program.

Results. LIMMA and SAM identified genes potentially affected by the presence of AML, including homeodomain-interacting protein kinase 1 (HIPK1), mex-3 homolog D (MEX3D), and genes potentially affected by chemotherapy, including argininosuccinate synthase 1 (ASS1), notch homolog 1 (NOTCH1), zinc transporter ZIP6 (SLC39A6), and TP53-regulated inhibitor of apoptosis 1 (TRIAP1). The expression of 2 genes with potential biological significance in oral mucositis, ASS1 and SLC39A6 (alias LIV-1), was confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR).

Conclusions. Our results suggest that AML-specific deregulated immune responses and inflammatory tissue damage to the oral mucosa caused by chemotherapy may not be overcome by the natural cellular repair processes and therefore contribute to CIOM. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:446-452)

Mucositis, or inflammation of the oral and gastrointestinal mucous membranes, is common, often painful, and limits the applicable dose for chemotherapy.1 Severe mucositis can result in a delay or cessation in the delivery of cancer therapy.2 The incidence of chemotherapy-induced oral mucositis (CIOM) ranges from 30% to 75% depending on the chemotherapeutic regimen.3 In addition to pain, ulcerations associated with mucositis can become secondarily infected and greatly increase the risk of polymicrobial bacteremia, especially with streptococcal species.4

Risk factors for CIOM have been identified and include low body mass and certain chemotherapy agents (e.g., paclitaxel, doxorubicin, etoposide, 5-fluorouracil, and irinotecan).3,5 Increased risk from specific chemotherapeutic agents is likely the result of direct cytotoxic effects, including DNA damage. Other proposed risk factors include diagnosis of acute myeloid leukemia (AML), acute lymphocytic leukemia, or myelodysplastic syndrome; prolonged neutrophil recovery; young age; and conditioning regimen for bone marrow transplantation.6 However, the pathogenesis of CIOM is poorly understood and it is unclear why patients of the same age, malignancy, and chemotherapy regimens develop mucositis with different frequency and severity.7 CIOM demonstrates a wide variety of histologic changes ranging from degener-
ation of collagen to epithelial hyperplasia, the variability of which is likely a result of the biological complexity of mucositis.5

The main objective of this study was to compare up- and downregulation of gene expression related to the underlying disease state or chemotherapy regimen. We used gene expression profiling to determine molecular events of CIOM 48 hours following chemotherapy. The secondary objective was to validate key genes identified using quantitative real-time reverse transcriptase–polymerase chain reaction (qRT-PCR).

MATERIAL AND METHODS

Human buccal mucosa samples and RNA extraction

Five newly diagnosed AML patients (5 male, mean age ± SD, 58.6 ± 11.7 years) and 3 healthy control (HC) subjects (2 males, mean age ± SD, 51.7 ± 12.7 years, 1 female, age 44) were recruited under informed consent and approval by the Institutional Review Board at Carolinas Medical Center, Charlotte, NC. AML patients received standard induction chemotherapy: ara-C (cytarabine) 100 mg/m² for 7 days and daunorubicin 45 mg/m² every day for 3 days. Immediately before induction chemotherapy and 2 days following initiation of chemotherapy, buccal mucosa biopsy specimens, 3 × 2 mm in size, were collected. These serial biopsies were collected from the opposite side of the mouth. In addition, buccal mucosa specimens were collected from 3 HC subjects. All tissues were placed in an RNAlater solution (Ambion, Austin, TX) and stored at –80°C. Mucositis was evaluated by the World Health Organization mucositis scale (0-4),9 with grade of 3 or higher suggestive of severe mucositis. Patients were evaluated for mucositis every 2 days to discharge or day + 20.

RNA extraction, amplification, and GeneChip hybridization

Buccal mucosa specimens were homogenized in TRizol (Invitrogen Life Technologies, Carlsbad, CA) with a Brinkman Polytron tissue homogenizer. RNA was extracted, precipitated, purified with RNeasy columns (Qiagen, Valencia, CA), and quantified by UV absorbance. For the 5 AML patients treated (AML_Post-C group) or not (AML_Pre-C group) with chemotherapy, total RNA integrity for microarray analysis was determined by electrophoresis at 100 V for 45 minutes in ethidium bromide containing 1% agarose gel. Antisense biotinylated cRNA target probes were synthesized from total RNA according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). Target probes were individually hybridized to Human Genome U133 plus 2.0 Array (Affymetrix, Santa Clara, CA). Arrays were washed and stained with streptavidin-phycocerythrin (Molecular Probes, Eugene, OR) and scanned for fluorescence using the Agilent GeneArray Scanner G2500A (Agilent Technologies, Palo Alto, CA).

Analysis of microarray data

Data files, .CEL, were generated in GeneChip Operating Software (GCOS) (Affymetrix), using default settings for scaling and detection call parameters. The full microarray data set is in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) as series GSE10746. Robust Multichip Average (RMA) Express 1.0.4 program (Bolstad BM, University of California, Berkeley, CA) was used for background adjustment and quantile RNA normalization of the 54,675 probe sets encoding human genome transcripts. Prefiltering of data was performed to select for genes whose expression varied across the samples with SD/mean between 0.1 and 10.0 and called present in more than 25% of total samples resulting in a dataset of 32,685 probe sets. Further filtering was applied to ensure the absolute expression levels to be above background (signal intensity >100) for at least 3 samples. This step generated a data set of 6965 probe sets corresponding to 4838 unique genes. This data set was further processed using TM4/TMeV v4.5.1 program (http://www.tigr.org). Linear Models for Microarray data (LIMMA) were used for overall and pairwise comparison between the groups on RMA normalized signal intensities for the 6965 filtered probe sets. Significance analysis of microarrays (SAM) (1000 permutations) was applied to log₂ transformed data of the filtered dataset to compare HC, AML_Pre-Chemotherapy (AML_Pre-C) and AML_Post-Chemotherapy (AML_Post-C) groups.

qRT-PCR

Following microarray analysis, integrity of the RNA stored at –80°C was reevaluated with integrity gels using AMRESCO Elementary Gel kit (AMRESCO, Solon, OH). Full Spectrum Complete Transcriptome RNA amplification kit (System Biosciences, Mountain View, CA) was used to generate and linearly amplify the complementary DNA (cDNA) for each RNA sample that was extracted from buccal punch biopsies of AML patients and healthy control subjects, and for the XpressRef Human Universal Total RNA (SuperArray Bioscience Corporation, Frederick, MD). Resulting cDNAs were electrophoresed on a 3% agarose gel and bands were visualized with ethidium bromide.

To determine the optimal concentration of cDNA to be used in subsequent real-time PCR assays, an initial qRT-PCR assay was performed in 25-μL reaction vol-
ume for each selected primer set with serial dilutions of the cDNA amplified from the XpressRef Human Universal Total RNA and by using the RT2 Real-Time SYBR Green PCR Master Mix (SuperArray Bioscience Corporation, Frederick, MD). The XpressRef Human Universal Total RNA was included 6 times per run to take into account experimental variability and to normalize qRT-PCR data. The delta-delta Ct (ΔΔCt) method was used to determine fold changes in expression. This method is based on the difference between the points at which the fluorescence crosses the threshold called the Ct-values of a reference gene (invariable) and a test gene (variable), respectively, and in a logarithmic scale. To apply the ΔΔCt method for proposed qRT-PCR validations, we identified RPL13A (ribosomal protein L13a) as the housekeeping gene with the lowest coefficient of variance (CV = SD/Mean = 3%) across the samples. To provide a reference for changes in expression of selected genes, qRT-PCR was performed with the Cepheid SmartCycler System (Cepheid, Sunnyvale, CA) using the RT2 PCR Primer Set for RPL13A (SuperArray Bioscience Corporation). This assay was done in triplicate for each AML patient or healthy control cDNA sample using the cDNA obtained from the XpressRef Human Universal Total RNA as a positive control and RT2 Primer sets for each of the selected genes: ASS1, SLC39A6 (alias LIV-1), and the housekeeping gene RPL13A. To determine fold changes in expression, the formula $2^{\Delta \Delta Ct}$ was used, as the amplification efficiency was 100% for both the reference and test genes.

RESULTS

Using the Affymetrix Human Genome U133 plus 2.0 Array, gene expression profiling was completed for buccal mucosa biopsies collected from 5 AML patients, including samples from AML_Pre-C group (n = 4) and AML_Post-C group (n = 4), and those collected from HC subjects (n = 3). For 2 AML patients, RNA integrity was not acceptable for either the AML_Pre-C sample or the AML_Post-C sample. Four of the 5 AML patients developed oral mucositis. All biopsy sites healed well and did not develop an area of mucositis at the site.

AML_Pre-C and AML_Post-C samples were not perfectly matched, as for 2 AML patients either the AML_Pre-C or AML_Post-C buccal mucosa biopsies could not be processed because of insufficient RNA integrity. Thus, we simultaneously compared the 3 groups, HC (n = 3), AML_Pre-C (n = 4), and AML_Post-C (n = 4), using LIMMA and SAM with this small microarray dataset ($N_{Total} = 11$).

LIMMA and SAM analyses

We determined differentially expressed genes among the HC, AML_Pre-C, and AML_Post-C groups using LIMMA and SAM. A total of 967 probe sets corresponding to 779 unique genes (significance $P < .05$) and 272 probe sets corresponding to 229 unique genes (significance $P < .01$) were determined to be differentially expressed by LIMMA. A list of 13 significant genes among 229 found significant with a cutoff $P$ value less than .0001 in at least 1 pairwise comparison is presented in the “a” section of Table I. Overall, fold changes (FC) in expression were small to moderate (FC $= 1.2-3.5$). The LIMMA adjusted $P$ value was less than .05 for most of these 13 genes in at least 1 pairwise comparison (Table I, “a” section). Most genes with a LIMMA significance $P$ value less than .0001 in at least 1 pairwise comparison are significant with adjusted $P$ value less than .05 in the same pairwise comparison. No other gene from the less stringent probe set list of 779 unique genes mentioned earlier had a LIMMA adjusted $P$ value less than .05.

Five genes were found to be more likely affected by the presence of AML: neuroblast differentiation-associated protein (AHNACK); homeodomain-interacting protein kinase 2 (HIPK2); la ribonucleoprotein domain family, member 1 (LARP1); mex-3 homolog D (MEX3D); and retinol-binding protein 1 (RBP1), as they were up- or downregulated in both AML_Pre-C and AML_Post-C groups (Table I, “a” section).

Eight genes were found more likely to be affected by chemotherapy: argininosuccinate synthase 1 (ASS1), casein kinase 1 alpha 1 (CSNK1A1), dUTP pyrophosphatase (DUT [2 probe sets]), glioblastoma-amplified sequence (GBAS), heterogeneous nuclear ribonucleoprotein A0 (HNRPA0), hypothetical protein MGC5370 (MGC5370), TP53-regulated inhibitor of apoptosis 1 (TRIAP1), and transmembrane 7 superfamily 3 (TM7SF3), as they were only up- or downregulated in both AML_Pre-C and AML_Post-C (Table I, section “a”).

SAM generated a list of 249 significant probe sets (205 unique genes) with q less than 10%. The number of probe sets (q < 10%) in common with LIMMA (significance $P < .05$) was 171 corresponding to 143 unique genes. A total of 10 probe sets (q = 0%) was found to be in common with the list of 272 probes sets corresponding to 229 unique genes (significance $P < .01$) generated by LIMMA (Table II). The list includes MEX3D, likely to be affected by the presence of AML and 9 genes ASS1, DUT, family with sequence similarity 134, member A (FAM134A), GBAS, MGC5370, NOTCH1, SLC39A6, TRIAP1, and zinc finger protein 639 (ZNF639) likely to be affected by chemotherapy. Among these latter, FAM134A, NOTCH1, SLC39A6, and ZNF639 were not found significant, considering
more stringent significance $P$ value less than .0001 as determined by LIMMA (i.e., $P < .01$) (Tables I and II).

In addition, AHNAK, HIPK2, LARP1, RBP1, CSNK1A1, DUT (1 of the 2 probe sets), HNRPA0, and TM7SF3 were not found significant by SAM at the significance level $q = 0\%$ (Tables I and II). Following chemotherapy, the most differentially expressed genes (i.e., in terms of FC in expression) were ASS1 (down-regulated), with an average FC $\sim 3$, and SLC39A6 (upregulated), with an average FC $\sim 3$, compared with HC and AML_Pre-C groups. We validated these 2 genes using qRT-PCR as an independent technique. Fold changes in expression AML_Pre-C versus HC, AML_Post-C versus HC, and AML_Post-C versus AML_Pre-C were determined using the comparative delta-delta Ct method. Using qRT-PCR, ASS1 was

**Table I.** List of differentially expressed genes among HC ($n = 3$), AML Pre-C ($n = 4$), and AML Post-C ($n = 4$) groups ($N_{total} = 11$), as determined by LIMMA.

<table>
<thead>
<tr>
<th>No.</th>
<th>Probe set</th>
<th>Genename/putative function</th>
<th>HC vs Pre-C</th>
<th>HC vs Post-C</th>
<th>Pre-C vs Post-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC/P value</td>
<td>FC/P value</td>
<td>FC/P value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HC vs Pre-C</td>
<td>HC vs Post-C</td>
<td>Pre-C vs Post-C</td>
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<tr>
<td>1</td>
<td>211986_at</td>
<td>AHNAK</td>
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<td>0.8/2.7E-05</td>
<td>0.9/3.2E-02</td>
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<tr>
<td>2</td>
<td>207076_s_at</td>
<td>Argininosuccinate synthetase 1 [ASS1]</td>
<td>1.2/8.7E-02</td>
<td>0.4/2.2E-04</td>
<td>0.3/3.1E-05</td>
</tr>
<tr>
<td>3</td>
<td>1556007_s_at</td>
<td>Casein kinase 1, alpha 1 [CSNK1A1]</td>
<td>1.4/4.2E-02</td>
<td>2.2/4.0E-06</td>
<td>1.6/4.4E-04</td>
</tr>
<tr>
<td>4</td>
<td>208956_x_at</td>
<td>dUTP pyrophosphatase [DUT]</td>
<td>1.2/1.5E-03</td>
<td>0.8/6.7E-04</td>
<td>0.7/2.4E-05</td>
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<tr>
<td>5</td>
<td>209932_s_at</td>
<td>dUTP pyrophosphatase [DUT]</td>
<td>1.1/6.5E-02</td>
<td>0.8/2.7E-03</td>
<td>0.7/8.4E-06</td>
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<td>6</td>
<td>201816_s_at</td>
<td>Glioblastoma amplified sequence [GBAS]</td>
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<td>0.8/7.8E-04</td>
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<td>0.8/7.4E-04</td>
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<td>1.8/5.3E-06</td>
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<td>Transmembrane 7 superfamily member 3 [TM7SF3]</td>
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<td>1.1/5.3E-01</td>
<td>1.9/1.3E-06</td>
<td>1.8/1.1E-06</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; HC, healthy control; LIMMA, linear models for microarray data; Post-C, postchemotherapy; Pre-C, prechemotherapy.

Genes that are up- or downregulated in both HC versus Pre-C and HC versus Post-C (with lowest $P$ values) but not Pre-C versus Post-C (with highest $P$ value) are likely affected, owing to the presence of AML (AHNAK, HIPK2, LARP1, MEX3D, and RBP1). Genes that are up- or downregulated in both HC versus Post-C and Pre-C versus Post-C (with lowest $P$ values) but not HC versus Pre-C (with highest $P$ value) are likely affected by chemotherapy (ASS1, CSNK1A1, DUT [2 probe sets], GBAS, HNRPA0, MDM2, TRIAP1 and TM7SF3).

*Affymetrix probe set identification.

*Gene name/putative function Affymetrix Human Genome U133 plus 2.0 Array annotation file.

*A fold change (FC) above 1 indicates upregulation, and an FC below 1 indicated downregulation.
confirmed to be downregulated with an average FC of approximately 4 and SLC39A6 to be upregulated with an average FC of approximately 2, in the AML_Post-C group compared with HC and AML_Pre-C groups.

Although qRT-PCR probes generally do not map to the target sequence of Affymetrix probe sets, Pearson correlation ($r^2$) between microarray and qRT-PCR data were 99.9% for ASS1 and 93.9% for SLC39A6. Similar to the results obtained by LIMMA, the results obtained by SAM also suggest that DNA-damage and inflammatory processes that trigger molecular feedback responses occur in the oral mucosa of chemotherapy-treated AML patients.

**DISCUSSION**

Our findings are consistent with a role for chemotherapy in gene expression changes with biological changes consistent with the onset of oral mucositis. AML patients who were treated with ara-C (cytarabine) 100 mg/m$^2$ for 7 days and daunorubicin 45 to 60 mg/m$^2$ every day for 3 days demonstrated significant changes in global gene expression in buccal mucosa biopsies 48 hours after chemotherapy. Despite the small sample size of our AML study, LIMMA and SAM produced overlapping but different lists of significant genes with potential biological significance in CIOM. Thus, the results presented here have to be considered with caution and will be the subject of future validation studies. This said, 2 genes, with expression determined by LIMMA and SAM to be particularly affected 2 days after chemotherapy in AML patients (n = 4), were confirmed by qRT-PCR. The selected and confirmed genes ASS1 and LIV-1 are discussed in the following sections in the context of free radical and DNA dam-
age–induced apoptosis and systemic tissue injury in CIOM.

**Argininosuccinate synthase 1**

ASS1 is a rate-limiting factor for nitric oxide synthesis that provides a source of arginine via recycling of citrulline to inducible nitric oxide synthase (iNOS). It has also been shown that nitric oxide (NO) induces TP53 activation in cancer-associated chronic inflammation in response to DNA damage. ASS1 is coinduced with iNOS by lipopolysaccharide (LPS) and interferon gamma, whereas iNOS may be stimulated by proinflammatory cytokines, such as interleukin-6 that has been implicated in CIOM.

In the present study, the approximately threefold downregulation of ASS1 following chemotherapy (Table I) may be the result of repression by accumulation of arginine (or a close metabolite) caused by the pyrimidine antagonist ara-C. In mammalian cells, pyrimidine and arginine biosynthetic pathways are coregulated via their common precursor carbamyl-phosphate. As any disturbance in one pathway may affect the other, thus downregulation of ASS1 may lead to insufficient cellular levels of NO that contribute to apoptosis. According to our data, iNOS, which could be protective under NO-deprivation conditions, is not induced.

**Solute carrier family 39 (zinc transporter), member 6 (SLC39A6)**

SLC39A6 (alias LIV-1), upregulated approximately threefold in AML patients following chemotherapy compared with the HC and AML_Pre-C groups, plays an important role in epithelial–mesenchymal transition (EMT). During embryologic development, EMT involves genes responsible for tissue remodeling and wound repair. As no upstream or downstream gene target is associated with LIV-1 expression in EMT, such as signal transducer and activator of transcription 3 (STAT3), E-cadherin, or twist homolog 1 are differentially expressed in the current study. It is unclear what mechanism causes LIV-1 to be upregulated, and to what extent it will affect the integrity of the oral mucosa. Indeed, LIV-1 is known to be coregulated by estrogen along with trefoil factor family 1 (TFF1). TFF1 was previously shown to protect the gastric mucosa from inflammatory insults mediated by tumor necrosis factor-alpha, to stabilize the mucus layer, and to affect healing of the epithelium.

This study has a number of limitations, such as a small sample size. Next, we acknowledge that any inference on anticipated impact on protein levels and function will require validation in cell culture or animal models in future studies. Additionally, there are numerous subtypes of AML that may have different types of gene expression changes. Therefore, larger genetic studies taking into account these subtypes are needed to expand our understanding of this complex topic. Peripheral blood in these types of genetic studies is much easier to obtain than tissue biopsies. Further studies to compare how closely related the gene expression changes in peripheral blood are to the actual tissue changes is necessary to confirm the relevance of peripheral blood samples as a surrogate to genetic alteration at the tissue level. In addition, it is of particular interest to determine why some patients do not develop CIOM, despite initial damage caused by chemotherapy, such as 1 patient in our cohort. Indeed, gene expression profiles were similar except for 1 gene, sel-1 suppressor of lin-12-like (SEL1L), alias inflammatory bowel disease 2 (IBD2), that was upregulated as determined by differential ANOVA (data not shown).

Although our findings are preliminary and need to be validated by alternative approaches in cell culture or animal models and for a large cohort of AML patients, we identified gene expression difference that may play a role in CIOM. Future studies aimed at better characterization of protective mechanisms in the oral mucosa, using molecular and global gene expression approaches, will be important to develop prophylactic means to prevent CIOM. In particular, investigation of AML-specific immune deregulation and postchemotherapy tissue repair and anti-inflammatory responses in AML patients who experience CIOM compared with those who do not, will be critical to develop new therapies for this dose-limiting side effect of cancer treatment.

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**REFERENCES**


Reprint requests:
Michael T. Brennan, DDS, MHS
Department of Oral Medicine
Carolinas Medical Center
Charlotte, NC 28232
mike.brennan@carolinas.org