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BIOLOGY CONTRIBUTION

GENE EXPRESSION CHANGES IN CERVICAL SQUAMOUS CELL CARCINOMA AFTER INITIATION OF CHEMORADIATION AND CORRELATION WITH CLINICAL OUTCOME

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Purpose: **The purpose of this study was to investigate early gene expression changes after chemoradiation in a human solid tumor, allowing identification of chemoradiation-induced gene expression changes in the tumor as well as the tumor microenvironment. In addition we aimed to identify a gene expression profile that was associated with clinical outcome.**

Methods and Materials: **Microarray experiments were performed on cervical cancer specimens obtained before and 48 h after chemoradiation from 12 patients with Stage IB2 to IIIB squamous cell carcinoma of the cervix treated between April 2001 and August 2002.**

Results: **A total of 262 genes were identified that were significantly changed after chemoradiation. Genes involved in DNA repair were identified including DDB2, ERCC4, GADD45A, and XPC. In addition, significantly regulated cell-to-cell signaling pathways included insulin-like growth factor-1 (IGF-1), interferon, and vascular endothelial growth factor signaling. At a median follow-up of 41 months, 5 of 12 patients had experienced either local or distant failure. Supervised clustering analysis identified a 58-gene set from the pretreatment samples that were differentially expressed between patients with and without recurrence. Genes involved in integrin signaling and apoptosis pathways were identified in this gene set. Immortalization-upregulated protein (IMUP), IGF-2, and ARHD had particularly marked differences in expression between patients with and without recurrence.**

Conclusions: **Genetic profiling identified genes regulated by chemoradiation including DNA damage and cell-to-cell signaling pathways. Genes associated with recurrence were identified that will require validation in an independent patient data set to determine whether the 58-gene set associated with clinical outcome could be useful as a prognostic assay.** **© 2008 Elsevier Inc.**

Radiation, Cervical cancer, Gene expression, Microarray, DNA damage, Radiation response, Prognosis.

INTRODUCTION

Randomized trials have demonstrated that concurrent chemoradiation therapy is currently the most effective treatment for patients with locally advanced cervical cancer, curing approximately 80% of patients with Stage IB2 to IIB disease and 60% of patients with Stage III to IVA disease (1–3).

Further improvements in outcomes are likely to come from more accurate prediction of prognosis, allowing individualized intensified treatment, as well as from better insight into molecular mechanisms of treatment resistance with the goal of developing targeted radiosensitizers. Genome-wide expression profiling has the potential to aid in accomplishing both of these aims.

Radiation-induced gene expression changes have been previously investigated *in vitro* in cell lines as well as *in vivo* in lymphocytes, but to our knowledge this is the first report of gene expression profiling in a human solid tumor. Classically the genes identified as regulated by radiation include genes involved in DNA repair, cell cycle arrest, and growth control (4–10). These pathways as well as others were regulated in lymphocytes from patients undergoing total body irradiation for hematologic cancers (11). However gene expression changes in tumor microenvironment, which has been shown to be an important determinant of radiation response (12–14), can best be appreciated by analysis of a solid tumor.

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Biologic predictors of outcomes in cervical cancer have been identified through a candidate gene approach as well as through microarray analysis. Proteins that have been shown to correlate with clinical outcome using immunohistochemistry include Ku80, GADD45, bax, bcl-2, intracellular adhesion molecule–3 (ICAM-3), and hypoxia inducible factor (HIF)-1α (15–21). Microarray analysis has been performed on cervical cancer–derived cell lines, comparing resistant to sensitive cells lines, as well as on patient samples, comparing patients with favorable to unfavorable clinical outcomes (4, 22–27). In these previous studies, sets of genes associated with pathways such as apoptosis, angiogenesis, and tumor cell invasion were found to be predictive of outcome. However, to our knowledge, no studies to date have investigated genes associated with clinical outcome after chemoradiation, the current standard of care for locally advanced cervical cancer.

In addition, genome-wide expression analysis could identify novel pathways that affect radiation sensitivity and resistance. Investigating genes associated with prognosis as well as chemoradiation-induced gene expression changes may identify novel pathways that protect tumor cells from treatment effects.

With the goal of identifying genes associated with outcome as well as genes regulated by chemoradiation in cervical cancer, we conducted a prospective pilot study in which we investigated gene expression in paired human cervical cancer specimens obtained before and shortly after the initiation of chemoradiation.

METHODS AND MATERIALS

Our institutional review board approved this prospective pilot study and all participants provided written informed consent. The study was open to women with International Federation of Gynecology and Obstetrics (FIGO) Stage IB2 or IIA cervical cancer with tumors >4 cm and patients with Stage IIB or IIIB squamous cell carcinoma of the cervix. All patients were treated with radiation and weekly cisplatin or cisplatin/5FU given every 3 weeks. A dose of 45 Gy was delivered to the pelvis in 25 fractions of 180 cGy over 5 weeks using an AP/PA technique and 15- or 18-MV photons. Within a week after completion of external-beam radiation therapy, two low-dose-rate intracavitary brachytherapy treatments were given 14 days apart with an after-loaded tandem and ovoid system. The final chemotherapy treatment (cisplatin alone with or without 5-fluorouracil) was given at the time of the second brachytherapy treatment. Two transvaginal punch biopsies were performed, before and after approximately 48 h from the start of treatment. At the time of the second biopsy, all patients had received two or three external beam treatments and the first dose of chemotherapy.

Microarray analysis

Cervical cancer biopsies obtained for the purpose of this study were immediately immersed in RNAlater tissue collection and RNA stabilization solution (Ambion, Austin, TX). Tumor samples were homogenized in Trizol reagent (Invitrogen) using a Pro 200 (Pro Scientific, Oxford, CT) homogenizer. Total cellular RNA was isolated according to the manufacturer's protocol. The precipitated total RNA was resuspended in diethylpyrocarbonate-treated water. Contaminating DNA was removed using a DNA-Free kit (Ambion). RNA samples were analyzed for RNA integrity with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Seventeen pairs of tissue specimens were obtained, but because of unsatisfactory RNA quality in some specimens, only 12 pairs of specimens could ultimately be analyzed from the pre- and post-treatment samples. For the change in gene expression analysis, 10 pairs of samples had adequate RNA for microarray analysis.

The cDNA was prepared as described previously (28). Hybridization to microarrays was performed using human oligonucleotide–spotted glass array with 18,861 60-mer oligos and controls produced in the Wiegand Radiation Oncology Microarray Core Facility at our institution. A reference RNA, universal human RNA (Stratagene, La Jolla, CA) was used in all experiments for normalization, and all experiments were conducted in duplicate. Hybridization was carried out for 16 h at 50°C. Slides were washed as previously described (28) and scanned with an ArrayWoRx autoscanner (Applied Precision, Issaquah, WA).

Quantified image data were processed using the statistical package Splus 6 (Insightful, Seattle, WA). Local estimates of background signal intensity were subtracted from raw total signal intensities for each feature. A logarithm-2 transformation was applied to the background-corrected signals. Within each channel, cy3 and cy5, on each array, the logarithm-2–transformed signals were normalized to the 75th percentile of the signal intensity. Signals were filtered using the requirement that the signal-to-noise ratio be >2 in at least 80% of the arrays in each group. The ratio of the logarithm-2–based data between the two samples was used for pathway analysis.

Statistical analysis

Two-group *t*-tests for dependent samples were used to identify genes with different expression in patients with and without recurrence. Multiple hypotheses testing was controlled by applying the false-discovery-rate algorithm using the beta-uniform mixture model (29, 30). All statistical analyses were performed using the S-PLUS-6 statistical software package (Insightful, Seattle, WA). Hierarchical clustering was performed using a rank correlation distance metric and average-linkage joining method.

Pathway analysis

To determine whether genes with differential expression interacted biologically and to identify the cellular pathways that differed most between the samples from patients with and without recurrence, we used Ingenuity Systems' Pathways Analysis software (Mountainview, CA; www.ingenuity.com). This software assigns genes to functional networks that are defined on the basis of functional relationships between gene products reported in the literature. The software then associates each network with a global cellular function (*e.g.,* cell cycle regulation) and with specific pathways comprising the network (*e.g.,* the IGF-1 pathway).

A data set containing all gene identifiers and the corresponding log ratio of expression between two groups and *p* values was entered into the Pathways Analysis software. Filtering criteria were set on the basis of the false-discovery-rate cutoff and log ratio values, and the filtered data were used for pathway analysis. Each gene identifier was mapped to a corresponding gene in the Ingenuity Systems Pathways knowledge base. The genes were overlaid onto a global molecular network developed from information contained in this knowledge base. Networks of genes were then generated according to the Pathways Analysis algorithms on the basis of their functional relatedness. The significance of the gene pathways was expressed as a *p* value, which was calculated using the right-tailed Fisher's exact