Feasibility of RNA collection for micro-array gene expression analysis in the treatment of cervical carcinoma: A scientific correlate of RTOG C-0128


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Received 2 December 2004
Available online 19 February 2005

Abstract

Purpose. To determine the feasibility of RNA collection in a multi-institutional cooperative group setting to be utilized for micro-array gene expression analysis, and to describe the methodology.

Methods. RTOG C0128, a phase I–II, protocol was designed to look at the safety and efficacy of external beam radiation therapy to 45 Gy with concomitant 5-FU and cisplatin chemotherapy, brachytherapy to deliver 85 Gy to point A, and Celecoxib at 400 mg twice daily for 1 year. Patients had the option of participating in a tissue collection portion of the protocol to be utilized for micro-array gene expression analysis before treatment and at the time of the first implant. RNA quality was determined by two parameters: the absorbance ratio at 260 nm/280 nm, and by the ratio of the integrated peak of 28S RNA to 18S RNA after gel electrophoresis.

Results. From August 2001 to March 2004, 84 patients were accrued to the trial, and tissue was obtained prior to initiation of therapy on 34 patients (40%). FIGO stages for the patients who provided tissue were IB (23%), II (57%), and IIIA–IVA (20%). Additionally, biopsies were obtained at the time of the first implant from 22 of the accrued patients making paired samples available on 26% for RNA extraction and micro-array gene expression analysis. The mean ± SEM amount of tissue obtained pretreatment was 97 ± 13 mg compared with 51 ± 8 mg for tissue obtained at the time of the first implant (P = 0.009). The mean total RNA extracted from the samples prior to treatment was 119 ± 19 μg versus 35 ± 6 μg at the time of the first procedure (P = 0.001). The RNA quality was assessed via the absorbance ratio at 260 nm divided by 280 nm. The mean values pretreatment and at first implant were 1.87 ± 0.07 versus 1.66 ± 0.11, respectively (P = 0.002); however, the integrated peak of 28S RNA to 18S RNA after gel electrophoresis was not significantly different (P = 0.26).

Conclusions. RNA extraction for gene expression analysis can be successfully performed in the multi-institutional cooperative group setting. Fresh tissue samples were obtained on 40% of accrued patients prior to treatment. The amount of biopsy material and the quantity of RNA extracted were greater prior to treatment compared with the first implant. The quality of RNA was superior prior to treatment as measured by the ratio of absorbance at 260/280 nm. These results indicate that gene expression analysis is feasible in the...
cooperative group setting utilizing amplification techniques for the RNA. Hopefully, this will allow for improvement in prognosis, therapeutic development, and correlation with acute and late toxicities in patients with cancer.

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Keywords: RNA; Cervix cancer; Radiation

Introduction

The treatment of advanced cervical carcinoma now entails the use of combined chemoradiotherapy. Phase III studies have demonstrated an overall survival benefit with the addition of cisplatin-based chemotherapy along with concurrent radiotherapy [1,2]. Otherwise, the treatment of advanced cervical cancer has changed little in the past 50 years. Standard prognostic factors include FIGO stage, tumor size, lymph node involvement, and histology. Molecular markers are not routinely used in placing patients into separate prognostic groups nor are specific tests used as predictive analyses to evaluate response. Cervix cancer is a good tumor site for correlative biologic studies because of relative ease of access to tumor in situ or the ease of biopsy compared to many other tumor sites.

Functional genomics is the study of gene function most commonly using microarray technology to evaluate gene expression. Functional genomics or microarray technology has been utilized in drug and target discovery, biomarker determination, toxicogenomics, chemotherapy sensitivity and resistance, and development of prognostic tests and disease subclass determination [3–8]. Microarray technology has been utilized to evaluate radiation responses to low dose radiotherapy and acute persistent responses to radiotherapy [9,10]. Additionally, microarray technology has been used to evaluate for late effects of radiation in model systems and has been utilized to evaluate for radiosensitivity in patients with carcinoma of the cervix [11–15]. For lesions of the cervix, gene expression microarrays have been used for HPV genotyping also [16,17].

The purpose of this study is to determine the feasibility of RNA collection in a multi-institutional cooperative group setting to be utilized for microarray gene expression analysis and to describe the methodology. In addition to prognostic information obtainable from cDNA microarray analysis from cervical tumor tissue prior to treatment, we are interested in response to chemoradiotherapy. Thus, biopsies were obtained prior to treatment and at the time of the first brachytherapy procedure.

Methods and materials

RTOG C0128 is a phase I/II study of COX-2 inhibitor, Celebrex (Celecoxib), and chemoradiation in patients with locally advanced cervix cancer. 5-FU and cisplatin chemotherapy were utilized according to the experimental arm of RTOG 9001 [1]. Celecoxib was administered at 400 mg twice daily for 1 year. The patient had the option of participating in a tissue collection portion of the protocol to be utilized for microarray gene expression analysis. Institutional Review Board consent was obtained at each institution. Patients gave written consent for participation in the therapeutic portion of the trial and for permitting tissue to be used for research purposes.

After consent was obtained, a biopsy of the cervix was performed with two to three passages of Tischler biopsy forceps prior to treatment. Fresh tissue was placed immediately into RNA later™ solution. The RNA later™ solution was previously supplied to RTOG institutions that had opened the protocol. Upon registration of patients onto the protocol, additional vials of RNA later™ were sent to the accruing institution from the RTOG tissue repository at LDS Hospital. Once the tissue was placed in RNA later™ solution, it was sent via overnight mail to the RTOG tissue repository. The tissue was divided with the large portion weighed and frozen in a small aliquot of RNA later™ and a small portion was placed into a paraffin block for histologic analysis. A hematoxylin and eosin slide was evaluated for presence of tumor, degree of necrosis, and grade.

RNA extraction was performed by the Trizol™ method, according to the manufacturer’s protocol. A polymerase chain reaction (PCR)-based amplification method was used to amplify message RNA (ribo amp™ kit). Briefly, the extracted total cellular RNA is transcribed to cDNA utilizing a T7 promoter in RNase free water. A second strand synthesis reaction using exogenous primers yields double-stranded cDNA. The cDNA is column purified, then transcribed using a T7 RNA polymerase. The antisense RNA is then purified.

Total RNA quality was evaluated by electrophoresis on a bioanalyzer™ and ratio of absorbance at 260 nanometers versus 280 nanometers was determined. The amplified RNA quality was again assessed on the bioanalyzer™ to evaluate the purity of the RNA. The ratios of the integrated 28S RNA peak to the 18S RNA peak were used as an indicator of RNA quality after amplification.

Once the amplified RNA was evaluated for quality, it was subsequently hybridized to a custom-made utilized gene chip manufactured at the Huntsman Cancer Institute. This gene chip contains greater than 25,000 known genes and ESTs. Thirty-four patients had tissue biopsies performed prior to the initiation of therapy while 22 patients had biopsies performed at the time of the first intracavitary implant.
Statistical analyses were performed on paired samples prior to treatment and at the time of the first brachytherapy procedure. The assumption of normality was checked for the difference between pretreatment and time of first implant for each variable. This assumption was not violated for total tissue volume, 28S/18S, and total RNA, and therefore the paired \( t \) test was used to test for differences in each of these variables. The assumption of normality was violated for 260/280 of RNA and therefore the non-parametric Wilcoxon sign rank test was used. All reported \( P \) values are with respect to the tests specified above. Differences were considered significant when the probability of error was below 5% (\( P < 0.05 \)).

Results

From August 2001 to March 2004, 84 patients were accrued to the trial, and tissue was obtained prior to initiation of therapy on 34 patients (40%). FIGO stages for the patients who provided tissue were IB (23%), II (57%), and IIIA–IVA (20%). Additionally, biopsies were obtained at the time of the first implant from 22 of the accrued patients making paired samples available on 26% for RNA extraction and micro-array gene expression analysis. Biopsies for microarray analysis were performed at 14 participating institutions. Eleven samples were obtained from 3 institutions each; one institution each provided 3, 4, and 5 samples; three institutions provided 2 samples each; and five institutions provided one sample. Because of the small number of samples in multiple groups, statistics were not performed by institution.

After extraction of total RNA, the amplified message RNA quality was assessed on the bioanalyzer to evaluate the purity of the RNA (Fig. 1). RNA samples were deemed adequate if their ratio of 28S/18S was \( >1 \). By this definition, 37/56 (66%) samples were adequate after amplification to proceed to microarray hybridization. Additionally, 36/56 (64%) of cases had a ratio of absorbance 260/280 nm greater than 1.7.

Biopsies were obtained from 22 patients at the time of the first brachytherapy procedure. Tumor was identified in only 11 cases, indicating that the chemoradiation had rendered a pathologic complete response in 11/22 (50%) of patients. Only 9 and 7 paired samples had grade or degree of necrosis, respectively, both evaluable. Given the limited sample sizes, statistical tests were not performed.

Comparisons were performed on paired samples prior to treatment and at the time of the first brachytherapy procedure (Fig. 2). The mean ± standard error of the mean (SEM) amount of tissue obtained pretreatment was 97 ± 13 mg compared with 51 ± 8 mg for tissue obtained at the time of the first implant (\( P = 0.009 \)). The mean total RNA extracted from the samples prior to treatment was 119 ± 19 \( \mu \)g versus 35 ± 6 \( \mu \)g at the time of the first procedure (\( P = 0.001 \)). RNA quality was assessed via the absorbance ratio at 260 nm divided by 280 nm. The mean values pretreatment and at first implant were 1.87 ± 0.07 versus 1.66 ± 0.11, respectively (\( P = 0.002 \)); however, the integrated peak of 28S RNA to 18S RNA after gel electrophoresis was not significantly different (\( P = 0.26 \)).

Discussion

Gene expression profiling by microarray analysis has been utilized for drug and target discovery, biomarker determination, toxicogenomics, chemotherapy and radiotherapy sensitivity, development of prognostic tests, disease subclass determination, and late effects of radiation in model systems [3–9]. Advanced cervical cancer is responsive to chemoradiation, and large tumors can be cured with radiation alone [1,2]. However, the high doses of pelvic radiation used result in a significant probability of radiation-induced late effects despite expertly applied brachytherapy [18]. Gene expression profiling by microarray may allow for an individual or “personalized” approach to patients with advanced cervical carcinoma. Harima et al. were able to identify a predictive score based on the expression of 35 genes that confer sensitivity to chemoradiation [13]. It is feasible that gene expression analysis may allow appropriate selection of chemotherapeutic agents or for radiation dose escalation protocols.
260/280 nm, the non-parametric Wilcoxon sign rank test was used. For total tissue volume, total RNA, and 28S/18S, the paired t test was used; while for absorbance at 260/280 nm, the non-parametric Wilcoxon sign rank test was used.

In our study, histologic analysis at the time of the first brachytherapy procedure yielded tumor in 50% (11/22) of patients. This is consistent with the known sensitivity of cervical cancers to radiotherapy. In one study, the median dose at which tumor was no longer clinically evident was 61.5 Gy (95% confidence interval: 50.7–72.3 Gy) or 42 elapsed treatment days [19]. Cervical carcinoma has been shown to respond faster to chemoradiotherapy compared to radiotherapy alone [20].

High-quality purified RNA is a requirement for successful application of microarray technology. Messenger RNA (mRNA) is unstable and subject to degradation particularly in the ex vivo state. RNA later™ was used to stabilize the RNA. Investigators were advised to place specimens immediately into the stabilizing solution. Previous studies have demonstrated good results utilizing RNA later™ [21]. Another common method for stabilizing mRNA is to flash freeze samples in liquid nitrogen. We found the utilization of RNA later™ to be convenient for the multi-institutional setting. Minimizing the time that the biopsy material is not frozen is likely important particularly before placement into RNA later™. In future studies, surgeons will be reminded to place the biopsy material immediately into the stabilizing solution and to avoid having the tissue contact skin to prevent mRNAse enzymatic degradation.

Amplification techniques for RNA have decreased the need for large tissue samples. In our study, adequate quantity of tissue and RNA was obtained with 2–3 passes with a Tischler biopsy forceps. The amount of biopsy material (\( P = 0.009 \)) and the quantity of RNA extracted (\( P = 0.001 \)) were greater prior to treatment compared with the first implant. This is likely due to the easier availability of obtaining tissue prior to the regression seen after chemoradiation. This is further highlighted by the fact that the initial biopsies were obtained in the office while the second biopsies obtained at the time of the first implant were performed under anesthesia. It is also possible that the reduction in RNA yield after the initiation of chemoradiation may be due to a decrease in cellularity of the specimens.

In this analysis, only 66% (37/56) of the amplified RNA samples were of adequate quality for RNA analysis. Given the logistics of a multi-institutional study, we are not deterred by this result. Additionally, we have established a rigorous criteria for sample adequacy by performing histologic analysis; and evaluation of RNA purity by absorbance at 260/280 nm and by gel electrophoresis.

In summary, RNA extraction for gene expression analysis can be successfully performed in the multi-institutional cooperative group setting. Fresh tissue samples were obtained on 40% (34/84) of accrued patients prior to treatment. These results indicate that gene expression analysis is feasible in the cooperative group setting utilizing amplification techniques for the RNA. Hopefully, this will allow for improvement in prognosis, therapeutic development, and correlation with acute and late toxicities in patients with cancer.

References


