Interferon-α Resistance Associated Genes in Renal Cell Carcinoma Identified by Expression Profiling

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Purpose: We identified differentially expressed genes associated with response to pegylated interferon-α treatment in patients with renal cell carcinoma.
Materials and Methods: We performed expression profiling on renal cell carcinoma specimens isolated from 23 patients with metastatic disease who were subsequently treated with interferon. Significance Analysis for Microarrays software was used to identify genes that were differentially expressed between patients with partial response compared to those with disease progression.
Results: A candidate gene approach looking at *VHL* and known target genes did not identify any genes whose expression correlated with patient response. A global analysis of approximately 54,000 probe sets identified 4 genes that had expression correlated with response. Reverse transcriptase-polymerase chain reaction analysis of 2 of these genes confirmed that they were more highly expressed in tumors from patients who responded to interferon-α. Interestingly, both of these genes mapped to 4q31-32, a region that has been implicated as the site of a potential tumor suppressor gene in renal cell carcinoma.
Conclusions: We have identified 4 genes (3 uncharacterized and 1 known) that may prove useful in predicting response to interferon-α treatment in patients with renal cell carcinoma.

Key Words: interferon-alpha; gene expression profiling; genes, tumor suppressor; carcinoma, renal cell

Renal cell carcinoma is the most common neoplasm of the kidney, with approximately 32,000 cases diagnosed each year in the United States.[1,2] RCC tumors present as distinct histological subtypes, namely clear cell, papillary and chromophobe subtypes.[1,2] Early stage disease is treatable with 5-year survival rates approaching 95%.[2] However, the survival rates for patients with metastatic disease are extremely low, with a 2-year survival rate of less than 20%.[2] This poor survival rate reflects the refractory nature of metastatic RCC to chemotherapeutic agents. Although new classes of drugs such as sunitinib and sorafenib that target receptor tyrosine kinases show great promise for the treatment of metastatic kidney cancer,[3,4] the current standard of care is treatment with cytokines such as high dose interleukin-2 or interferon-α. The response rates to these therapies are low, with only 10% to 20% of patients with RCC responding to IFN-α therapy.[2,5,6]

IFNs are glycoproteins that activate a complex series of cellular responses. IFNs can elicit their effects through antiproliferative, antiviral, antitumor and immunomodulatory activities.[7] A number of studies have attempted to identify genes and functional pathways associated with an IFN resistant phenotype in a variety of tumor types.[8,9] Many of these studies have been performed in vitro and, thus, any host or microenvironmental effects would be missed. Furthermore, these studies usually focus on changes in expression following IFN treatment rather than on preexisting gene expression differences that could predict response. As a result many of the genes involved in IFN resistance in RCC remain unknown.

We describe expression profiling on a panel of RCC tumors from patients with known response to IFN-α treatment. These patients were treated with a pegylated form of IFN-α-2b (PEG-Intron®), which is reported to have better stability and reduced toxicity compared to free IFN-α.[6] We report the identification of a small set of genes whose expression was associated with a clinical response to IFN-α.

MATERIALS AND METHODS

Patients

Tumor material was collected from a panel of 23 patients with metastatic RCC who had received PEG-Intron treatment at Memorial Sloan-Kettering Cancer Center between 2002 and 2004. Patient material was obtained under an institutional review board approved protocol. Fresh tissue was collected using standardized procedures with all specimens snap frozen in isopentane immediately after removal and processing. Of the 23 tumors 15 were from the primary

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site while the remaining 8 were from metastases. The clinical features of these patients and their clinical response are shown in the Appendix. Further description of these patients is being prepared for a separate report.

RNA Isolation

RNA was isolated from frozen tumor blocks as previously described.[10] Briefly, tumors were grossly dissected to exclude normal tissue, then minced and homogenized. RNA was purified through RNeasy columns (Qiagen, Valencia, California) according to manufacturer protocols. RNA quantity was assessed by ultraviolet absorbance, and RNA quality was verified on denaturing agarose gels by the presence of distinct 28S and 18S ribosomal bands. RNA from a patient with no evidence of kidney cancer was also extracted as a control.

cRNA Labeling and Hybridization

Probes for hybridization to Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, California) were prepared as described previously.[10] Briefly, 12 μg of total RNA was converted into double stranded cDNA using a T7 modified oligo dT primer and a ds cDNA synthesis kit (Invitrogen, Valencia, California). Biotinylated cRNA probe was produced from the ds cDNA template using a HighYield™ RNA transcript labeling kit (Enzo Biochem, Farmingdale, New York). The cRNA probe was quantitated by ultraviolet absorbance, and 15 μg were fragmented according to manufacturer protocols for hybridization to the U133 Plus 2.0 microarrays. Hybridization, washing and imaging were performed according to manufacturer protocols.

RT-PCR Analysis

RT-PCR was done as described previously.[10] Briefly, 1 μg of total RNA was reverse transcribed using random hexamers (Invitrogen) and superscript II reverse transcriptase (Invitrogen) in 50 μl total volume. cDNA products (1 μl) were then amplified using 35 cycles of 95C melting (45 seconds), annealing (30 seconds) and 72C extension (45 seconds), with gene specific primers: Hs.94122, 52C annealing, FWD: 5′-GGGCAATTTGTTGCTTTACAA-3′, REV: 5′-TCAACAGCAACGATATGACATT-3′; Hs.11325, 56C annealing, FWD: 5′-CCCTGAATTTGGTTTGCAGT-3′, REV: 5′-AGCCTCGTAATGCAAAAAGC-3′; *ACTB*, 56C annealing, FWD: 5′-ATCTGGCACCACACCTTCTAGAATGAGCTGCG-3, REV: 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. PCR products were visualized on 1% agarose gels.

Data Normalization and Statistical Analysis

Raw.cel files were normalized, background subtracted and log transformed as described previously.[10] Significantly differentially expressed genes were identified using SAM.[11] Candidate genes were tested for significance using the t test with Benjamini-Hochberg correction for multiple comparisons within Bioconductor for R.[12]

RESULTS

Expression profiling using Affymetrix U133 Plus 2.0 microarrays, which consist of 54,627 probe sets, was performed on 23 renal cell carcinoma specimens from patients with metastatic disease who were subsequently treated with IFN. Eight patients showed partial response, 9 had stable disease as the best response and 6 had overt disease progression with treatment. The characteristics of the tumors that were profiled are shown in the Appendix.

Expression of Genes Known to be Involved in RCC

A number of proteins have been previously implicated as being important in RCC, most notably VHL and its downstream targets HIF-1α and HIF-2α.[1] A number of genes have been identified through expression profiling and other studies that are regulated in response to changes in VHL or HIF levels, including *CA9*, *VEGF* and *CCND1*.[13,14] We examined the expression levels of 32 genes (90 probe sets) that have been implicated as target genes for correlation with response (progression of disease, stable disease and partial or moderate response). Unsupervised clustering of the tumor samples based on expression of these genes did not show any separation on the basis of response to IFN (fig. 1). Statistical analysis for differential expression comparing patients with partial response to those with stable disease and progression of disease showed 2 significant probe sets before correction for multiple comparisons (both GLS), with no significant probe sets following this correction. Similarly, comparison of patients with partial response or stable disease to those with progression of disease yielded 1 significant probe set (GLS) before the multiple comparison correction, but none following this correction.

Expression Differences Correlated With Clinical Response

We next examined expression differences in the entire gene set for correlations with response. An analysis of the full expression profiles of patients for therapeutic benefit (patients with PR/SD vs those with POD) identified 3 significantly differentially expressed genes (SAM estimated False Discovery Rate 0), consisting of Hs.76704 (Affymetrix probe set 226192_at), Hs.11325 (228046_at), and *ABCD3* (202850_at). Further analysis of just PR cases vs POD identified an additional expressed sequence tag [Hs.94122 (228423_at)] that was significantly differentially expressed (SAM estimated False Discovery Rate 0.33). The average expression of each of these genes within PR, SD and POD groups relative to normal kidney is shown in figure 2. All 4 shared a similar expression pattern with higher levels in the PR group and lower levels in the POD group. To ensure that these differences were not due to differences in primary compared to metastatic lesions, we compared expression of these 4 genes between the primary tumors and metastases. None of the genes showed significant differences in expression between these 2 groups.

Validation of Expression by RT-PCR

To validate the array results the 2 expressed sequence tags were chosen for RT-PCR analysis. Hs.11325 and Hs.94122 were analyzed within a subset of the tumor samples. Of these tumors 4 showed PR while the remaining 4 showed POD. As shown in figure 3 RT-PCR analysis confirmed the differential expression observed with the microarrays, as both genes were more highly expressed in patients with PR compared to those with POD.

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