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A gene signature associated with upregulation of the Wnt/B-catenin signaling pathway predicts tumor response to trans-arterial embolization

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Abstract

Purpose—To identify gene mutations in tumors undergoing trans-arterial embolization (TAE) and explore the relationship between gene mutations and tumor response to TAE.

Materials and Methods—This was a retrospective review that included 17 patients with primary or metastatic liver tumors treated with TAE and that had specimens analyzed for a 341-gene panel next generation sequence assay. Pathology included hepatocellular carcinoma, carcinoid, pancreatic neuroendocrine, melanoma, medullary thyroid, and liver acinar cell carcinoma. Disease, procedure data, and tumor response data were collected. Dimensionality reduction was performed using principal component analysis. A linear support vector machine was used to learn a prediction rule and identify the genes most predictive of objective tumor response (CR+PR) by mRECIST. Cross-validation was used to test the prediction on the hold-out set. Permutation testing was used to determine statistical significance of the prediction accuracy. Recursive feature elimination was used to identify the most predictive genes.

Results—Nine tumors were responders and eight tumors were non-responders based on mRECIST criteria at 4 months post-embolization. Using the top 2 principal components, prediction accuracy of the gene mutation signature was 70% (+/–11%), which was statistically significant ($p < 0.05$). The most predictive genes were *CTNNB1*, *MEN1*, and *NCOR1*—three genes associated with the Wnt/B-catenin and hypoxia signaling pathways.

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Conclusions—This study identifies gene mutations in tumors treated with TAE. A gene mutation signature obtained from the mutation data suggests that upregulation of the Wnt/B-catenin signaling pathway may be associated with sensitivity to TAE.

Introduction

Hepatic arterial embolization plays a central role in the management of unresectable primary and metastatic liver tumors. Significant survival benefit has been demonstrated in patients with hepatocellular carcinoma (HCC) undergoing chemoembolization (TACE) or embolization (TAE)¹; treatment efficacy has been demonstrated in other primary liver tumors and liver metastases.

Despite the success of embolization in many tumors, there remains wide variability in response in both HCC² and non-HCC³ tumors. The ability to predict tumor response to embolization remains an active area of research. Although tumor hyper-vascularity is often presumed to be associated with treatment response, pre-treatment cross-sectional imaging was not predictive of response in TACE of HCC and non-HCC tumor^{4,5}. Tumor type was not associated with outcome or response rate⁵. A randomized controlled trial comparing TACE and TAE in HCC demonstrated no statistically significant difference in outcome between the two groups, and comparable overall survival to previously reported studies, suggesting that the major mechanism of cell death for embolization is hypoxia⁶. But hypoxia induces a complex set of molecular changes that may promote cell death or cell survival⁷. The underlying genetic mechanisms determining response to hypoxia in embolization remain poorly understood.

Some progress has been made utilizing serum and tissue biomarkers to track tumor progression and response to embolization⁸⁻¹⁰. Recently genetic biomarkers predictive of HCC response have been reported¹¹⁻¹³. Despite these advances, it remains unknown which genetic markers are predictive of response in patients undergoing embolotherapy.

The purpose of this study was to identify the gene mutations in tumors undergoing TAE and explore the relationship between altered gene pathways and sensitivity or resistance to hypoxia induced by TAE.

Materials and Methods

This was an observational cohort study that was approved by the institutional review board with informed consent waived and was compliant with the Health Insurance Portability and Accountability Act. A retrospective review of a single institutional database identified consecutive patients from 2009-2015 (n=637) that underwent TAE of a primary or metastatic liver tumor. Only patients that also had molecular analysis performed of tissue specimens were included (n=86). Of these, only those that underwent the full institutional gene assay panel (n=17 patients, 18 tumors), which includes a written informed consent for genetic profiling, were included. One patient had genetic analysis performed on two separate biopsies performed on two distinct sites of TAE. Only the first tumor was included in subsequent statistical analysis (see below). In 17/18 cases, genetic analysis was performed

on the liver biopsy specimen and in 1/18 case genetic analysis was performed on the primary site of the tumor.

Tumor specimens from biopsies were formalin-fixed and paraffin-embedded. Genetic mutation analysis was performed using a previously reported hybridization capture-based next-generation sequencing assay for targeted deep sequencing of all exons and selected introns of 341 key cancer genes (see Supplemental Figure 1)¹⁴.

The decision to perform TAE was made by the interventional radiologist in conjunction with the medical oncologist and surgeon that form the hepato-biliary tumor disease management team at our institution. TAE was performed under general anesthesia or moderate sedation based on the interventional radiologist performing the procedure. All procedures were performed by a fellowship-trained interventional radiologist with at least 6 years of experience at the beginning of the study. TAE was performed as selectively as possible, treating individual segmental arteries when feasible and using either 40-120 and/or 100-300 micron beads (Embosphere Microspheres) until stasis was achieved (defined as technical success).

Imaging follow-up included either dedicated liver CT or liver MRI at 1 month after the procedure and then at 3-month intervals thereafter. Tumor radiologic response was assessed based on follow-up imaging using mRECIST criteria and categorized as either complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Objective response (OR) was defined as achieving CR or PR at four-month follow-up (mean 125 days, range 21-169 days); tumors were designated non-responders (NR) otherwise.

Statistical Analysis

Oncoprint data included all 18 tumors. Correlation with tumor response was performed on only 17 tumors--one patient had two tumors sequenced, only the first tumor mutation data and response were included. Dimensionality reduction was performed using principal component analysis, a standard multivariate statistical technique that allows a set of correlated variables to be reduced into a smaller number of uncorrelated variables which are made up of linear combinations of the original variables.¹⁵ The principal components are ordered such that the first principal components describe most of the variance in the data. To identify a predictive signature of TAE response, a linear support vector machine (SVM) was used—a technique that empirically separates two classes from each other in a high-dimensional feature space. Ten-fold cross-validation was used to estimate the classification error.¹⁶ To approximate the statistical significance of the classification error, non-parametric permutation testing was used¹⁷. Specifically, the class labels (OR and NR) were randomly permuted multiple times and the cross-validation was performed on each new permuted dataset, thus constructing an empirical cumulative distribution over the classification test errors. Recursive feature elimination (RFE) was used to identify the subset of mutations that were the most discriminative¹⁸. All source code was written in Matlab, and a freely available Matlab-implementation of SVM and RFE was used.¹⁹

Results

There were 18 tumors (from 17 patients) between 2009 and 2015 from either primary or metastatic liver tumors who underwent TAE and had complete genetic analysis performed on the tumor specimens. Average patient age was 61.7 (range 35-94). There were 13 males and 4 females. The median time between biopsy and embolization was 1 month (range 0 to 45 months). There were 3 patients with greater than 12 months interval between biopsy and embolization. Tumors included the following histology: 8 hepatocellular carcinoma (hcc) including two from the same patient, 4 carcinoid including lung, kidney, rectal, and ileal (car), 2 pancreatic neuroendocrine (pne), 2 melanoma (mel), 1 medullary thyroid (mth), and 1 liver acinar cell (lac). One patient underwent two embolization procedures on two different hcc tumors. Nine of the tumors were OR and nine of the tumors were NR based on criteria described in the Methods section. The patient that underwent embolizations and gene analysis on two different sites had an initial tumor that was labeled OR and second tumor that was labeled NR. The mean largest tumor size was 5.3 cm (range, 2.1 to 9.2 cm). For the 8 hcc tumors, the average alpha-fetoprotein level was 96.7 (range, 2.2 to 685). For the 6 neuroendocrine tumors, there were 2 low grade and 4 intermediate grade tumors.

Figure 1 summarizes genetic alterations (at least two mutations or copy number aberrations) identified in the cohort.²⁰ Principal component analysis was performed to identify trends in the data (see Figure 2). For the patient with two treated tumors with mutation analysis, the second treatment site was not included in this and subsequent analysis. Each tumor was mapped onto a 341-dimensional gene mutation space. Figure 2 displays the top three principal components, which correspond to the directions of greatest variance in the data. There is some clustering evident that is based on histology (see Figure 2a). For example, pne and car tumors cluster together and the two mel tumors are adjacent. In Figure 2b colors denote label assignment based on mRECIST criteria. Responders (OR) and non-responders (NR) appear to also cluster separately. To quantify whether OR and NR were distinguishable in gene mutation space, a linear SVM was performed using the top 2 principal components as input space with standard leave-one-out cross-validation. Prediction (testing) accuracy on the holdout set was 70% (+/- 11%). To estimate a confidence threshold on the testing accuracy, a permutation test was performed and found that this accuracy was statistically significant ($p < 0.05$).

To identify the most predictive genes recursive feature elimination was performed. The top 3 discriminative genes identified were *CTNNB1*, *MEN1*, and *NCOR1* (see Table 1). Repeating the linear SVM using just these 3 genes yielded a prediction accuracy of 85% (+/- 8%). *CTNNB1* and *MEN1* mutations were associated with good response to TAE and *NCOR1* was associated with poor response. All three mutations are associated with both the Wnt/B-catenin signaling pathway and the hypoxia signaling pathway (see Table 1). *CTNNB1* and *MEN1* mutations are associated with up-regulation of the Wnt/B-catenin signaling pathway and *NCOR1* is associated with down-regulation of this pathway. A closer analysis of the mutations of the *CTNNB1* gene show that all of the mutations occur on exon 3 at the GSK-3b phosphorylation sites (see Figure 3).²¹ Five of these mutations are known recurrent hot-spot mutations.²²

Discussion

Response to TAE for HCC and non-HCC tumors is variable and current clinical and imaging predictive markers are unreliable. This manuscript presents an initial exploratory study of gene mutations in tumors undergoing HAE and presents an approach to identify a gene signature predictive of tumor response to TAE based on the gene mutations. A subset of genes was identified that were the most predictive (*CTNNB1*, *MEN1*, *NCOR1*) of response.

The predictive genes identified raise some plausible mechanistic explanations regarding signaling pathways associated with response to TAE. All 3 genes are associated with the Wnt/B-catenin and hypoxia signaling pathways (see Table 1). Cross-talk between the Wnt signaling pathway and hypoxia signaling pathway is well-established.²³ This same pathway is known to directly upregulate glycolysis²⁴—addition to this upregulation (analogous to the concept of oncogene addiction²⁵) would make this pathway susceptible to embolization. But whereas *CTNNB1* and *MEN1* are positively associated with Wnt/B-catenin signaling, *NCOR1* is negatively associated. Specifically, *CTNNB1* is a downstream activator of the Wnt signaling pathway and *MEN1* promotes Wnt signaling. Meanwhile, *NCOR1* is a negative regulator of Wnt/B-catenin targets. Interestingly, this mirrors the finding where *CTNNB1* and *MEN1* mutations were associated with improved response and *NCOR1* mutations were associated with poor response. This suggests that up-regulation of the Wnt signaling pathway confers improved response to TAE—a plausible and testable hypothesis. Figure 4 summarizes the identified mutations in relation to the Wnt pathway.

An alternative hypothesis is that *CTNNB1* and *MEN1* are simply biomarkers of less aggressive disease. For example, Boyault et al identified 6 subgroups of HCC (G1-G6) and noted that G5 and G6 were associated with Wnt activation and potentially showed improved survival compared with G1-G3 groups²⁶. Similarly, in a cohort of pancreatic neuroendocrine tumors, Jiao et al demonstrated that *MEN1* mutations were associated with better prognosis²⁷. However, the clinical history of patients in both of these studies is unknown, and it may be that some of these patients also had undergone liver directed therapy.

Recently, Gaba et al analyzed 19 tumors in patients undergoing TACE using an mRNA expression assay of a panel of 60 pre-determined genes²⁸. They compared tumors with complete response and partial response and identified 11 genes with differential mRNA expression. Interestingly, six of the eleven significant and marginally significant genes they identified are associated with the Wnt/B-catenin signaling pathway: BAX²⁹, CCNE1³⁰, PP3CA³¹, SNX1³², and VEGFA^{33,34}, CXCL10³⁵. Further work to dissect the role of this signaling pathway in controlling response to embolization is warranted, potentially by combining our mutation level analysis with Gaba et al's expression level analysis.

The approach presented here differs from Gaba et al in several important ways. Specifically, (1) focus on response to TAE to identify a hypoxia specific signature, (2) use of gene mutation analysis rather than gene expression analysis and employment of next generation sequence techniques, (3) included a large panel of mutations, (4) focusing on a multivariate approach to take into account potential interactions, (5) employing machine learning

algorithms to identify a predictive gene signature that could predict tumor response, and (6) combining both HCC and non-HCC histologies to identify potentially common signaling pathways of hypoxia sensitivity.

The overall response rate was 50%. This was consistent even within tumor types that are known to respond to TAE, including hcc, car, and pne. The response rate in the cohort is likely biased, as only patients with tumors that underwent the 341-gene mutation analysis assay were included, and therefore, were more likely to include tumors that progressed. In the future, prospective collection of tissue specimens from patients undergoing embolization would result in less biased data.

In this retrospective analysis we used an institutional hybridization-capture next generation sequencing assay that enables detection of substitutions, duplications, insertions and deletions in a large number of cancer target genes in a single assay and with a well-established in-house bioinformatics pipeline. In the future with a larger dataset, other next generation sequencing methods may also be used including whole exome or whole genome sequencing³⁶.

There are several important limitations of this work. First, the cohort of patients with mutation analysis is small, so the distributions of responders and non-responders is under-sampled, making the learning task difficult and prone to over-fitting. Dimensionality reduction and cross-validation limit this problem, but the results must be interpreted cautiously as exploratory and needing validation. The cohort is heterogenous and therefore confounders such as tumor stage, disease burden, and tumor vascularity are difficult to control. Second, the investigated panel of 341 genes is limited so some relevant genes were likely not included. Third, the analysis was limited to mutation analysis and does not take into account changes at the level of transcription and translation. Fourth, the analysis included a combination of different tumor types. While in some cases mutations spanned multiple tumor types, there were also certainly differences in mutations between tumor types. Different tumor types have variable natural course, making comparison of response difficult. For example, neuroendocrine tumors can be slow growing, whereas melanoma metastases may grow very quickly. Fifth, gene mutations are dynamic both across space and time and three of our mutational analyses were based on specimens over a year prior to embolization³⁷. Finally, whether the results can be extrapolated to TACE is unclear.

This report presents an approach to identifying a gene signature predictive of tumor response to TAE based on gene mutation analysis. The results suggest an important role for the Wnt/B-catenin signaling pathway in this response and pose a testable hypothesis—“glycolysis addiction” is mediated by upregulation of this pathway and is associated with improved response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Genes with mutations and copy number aberrations identified in cohort of 18 tumors. Tumor types hepatocellular carcinoma (hcc), carcinoid (car), pancreatic neuroendocrine (pne), melanoma (mel), medullary thyroid (mth) and liver acinar cell (lac). Objective response (OR) and no response (NR). *Denotes second tumor embolized in same patient (not included in principal component analysis and learning algorithm).

Figure 2.

Top 3 principal components of gene mutation space. Each individual point represents a tumor. Tumors are colored by **(a)** histology and **(b)** response to TAE. Hepatocellular carcinoma (hcc), carcinoid (car), pancreatic neuroendocrine (pne), melanoma (mel), medullary thyroid (mth) and liver acinar cell (lac). Objective response (OR) and no response (NR).

Figure 3. CTNNB1 mutations mapped to the corresponding 781 amino acid protein. All of the mutations fall on exon 3 at the GSK-3b phosphorylation sites.

Figure 4. Summary of relationship between B-catenin, MEN1, and NCOR1 mutations and the Wnt signaling pathway that upregulates aerobic glycosylation.

Table 1

Top 3 predictive genes identified by recursive feature elimination. All 3 genes are associated with Wnt/B-catenin signaling and hypoxia signaling pathways.

Gene	Alias	Hypoxia	Wnt-signaling
CTNNB1	B-catenin	Enhances metastatic potential via HIF signaling	Downstream activator in Wnt signaling pathway
NCOR1	Nuclear receptor corepressor	Decreases PPAR activity, a suppressor of HIF signaling in cancer cells	Negative regulator of transcription targets of B-catenin
MEN1	Menin	Regulator of hypoxia stress response	Promotes Wnt signaling, interacts with B-catenin via nuclear shuttling