A Systems Biology Approach Identifies Effective Tumor-Stroma Common Targets for Oral Squamous Cell Carcinoma

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ABSTRACT

The complex interactions between cancer cells and their surrounding stromal microenvironment play important roles in tumor initiation and progression and represent viable targets for therapeutic intervention. Here, we propose a concept of common target perturbation (CTP). CTP acts simultaneously on the same target in both the tumor and its stroma that generates a bilateral disruption for potentially improved cancer therapy. To employ this concept, we design a systems biology strategy by combining experiment and computation to identify potential common target. Through progressive cycles of identification, TGF–β receptor III (TβRIII) is found as an epithelial–mesenchymal common target in oral squamous cell carcinoma. Simultaneous perturbation of TβRIII in the oral cancerous epithelial cells and their adjacent carcinoma-associated fibroblasts effectively inhibits tumor growth in vivo, and shows superiority to the unilateral perturbation of TβRIII in either cell type alone. This study indicates the strong potential to identify therapeutic targets by considering cancer cells and their adjacent stroma simultaneously. The CTP concept combined with our common target discovery strategy provides a framework for future targeted cancer combinatorial therapies.
INTRODUCTION

Tumors are mixtures comprising both neoplastic cells and stromal cells. It is becoming increasingly evident that the occurrence and development of tumors not only depend on the factors intrinsic to the tumor itself, but also require support from the stromal microenvironment surrounding the tumor (1). The complex interactions among tumor cells and the various types of cells and matrix elements within the microenvironment play important roles in cancer initiation, progression and invasion. For instance, carcinoma-associated fibroblasts (CAFs) are one of the most significant cell types found within the desmoplastic stroma in many types of carcinomas. CAFs interact with cancer cells through various signaling molecules, which can significantly affect cancer proliferation, survival, motility and invasion (2-4).

Decades of cancer research have identified a number of genes for anticancer treatment, from cancer cells as well as from stroma cells. The unilateral perturbation (ULP) strategy targeting these genes in either the tumor or its microenvironment alone has brought dramatic therapeutic advances and substantially improved the lives of cancer patients (5). However, cancer is extremely complex and the ULP treatment is only partially effective (6). Another combinatorial strategy, the bilateral perturbation (BLP), which targets both tumor and its microenvironment, has emerged as a promising candidate for cancer therapy by fighting against cancer as a whole system (7-9). However, the implementation of the BLP strategy is challenging. One bottleneck is the identification of multiple effective targets that each works in the tumor cells and the stromal cells.

It has been estimated that 5% to 10% of human genes may contribute to
tumorigenesis (10), with currently reported cancer genes accounting for only 1% of the human genome (11). The huge gap suggests that a large number of cancer genes remain to be identified. Although traditional gene mapping approaches, such as linkage analysis and association studies, have been proved to be powerful tools for disease gene discovery, they suffer from limited resolution to gene localization and great time and labor expense (12). In recent years, with the progress of high-throughput techniques and computational methods, systems biology approaches are being exploited to the discovery of disease genes and prognostic biomarkers (13-16). Systems biology provides unique tools to prioritize disease-related genes and identify potential target for cancer treatment.

In the present study, we introduced another concept called common target perturbation (CTP), which perturbs the same target in the cancerous epithelium and its adjacent stroma simultaneously to inhibit tumor growth. Without the multiple-targeting or potential complement between cancer and stroma, CTP provides a promise for improved cancer therapy. Key of the CTP concept is to identify effective tumor-stroma common targets, whose perturbation will be destructive for tumors. We test our CTP concept by performing simultaneous perturbation of oral cancerous epithelia cells and their adjacent CAFs using oral squamous cell carcinoma (OSCC) as a model system. We develop a systems biology strategy that combines experiment and computation for identification of the epithelial–mesenchymal common targets (EMCTs). These progressive organic cycles identified TβRIII (also known as TGFBR3) as a potential EMCT. In vivo assessment shows that the simultaneous perturbation of TβRIII in the cancerous epithelial cells and its adjacent CAFs effectively inhibited tumor growth, and that this CTP-derived treatment is more effective than the common ULP approach.
MATERIALS AND METHODS

Bioinformatics analysis. The cancer signature genes identified from samples of twenty cancer types were obtained from the work of Lu et al. (17). The human PPI data were downloaded from the Human Protein Reference Database. Three gene expression datasets (GSE6631 (36), GSE3524 (37), and GSE9638 (38)) of oral cavity tumors were downloaded from the Gene Expression Omnibus (GEO) database. Functional annotation of SPP1Net was completed using the DAVID webserver (39), and SPP1Net was drawn using Cytoscape (40).

Neighborhood analysis. To quantify the extent of gene expression changes in the cancerous epithelium and stroma with respect to their normal counterparts, we introduced a score: $f_{es,raw}$. The $f_{es,raw}$ score is defined as the absolute value of the product of the gene expression fold changes in the cancerous epithelium and stroma with respect to their normal counterparts. The higher $f_{es,raw}$ score of a gene will represent a higher extent of expression changes in both the cancerous epithelium and stroma, and thus a higher probability as an effective EMCT. We scaled the $f_{es,raw}$ score from 0 to 1 on the basis of the estimated cumulative density function derived from kernel-density estimation, so that the scaled $f_{es,raw}$ score of a gene, denoted as $f_{es}$, is the estimated percentage of genes with actual $f_{es,raw}$ scores below this gene. For a subnetwork $S$ of $k$ genes, we produced an aggregate score $f_S$ defined as the mean $f_{es}$ of all genes in the subnetwork:

$$f_S = \frac{\sum_{i \in S} f_{es,i}}{k}.$$ 

To determine whether the $f_S$ score of a subnetwork is higher than expected, we
randomly sampled gene sets of size $k$ independently of the network and computed their $f_S$ scores. We then used these to derive estimates for the score mean $\mu_k$ and standard deviation $\sigma_k$. Using these estimates, the normalized subnet score $Z_S$ is computed as:

$$Z_S = \frac{(f_S - \mu_k)}{\sigma_k}.$$ 

Based on the above scoring scheme, a Monte Carlo approach was implemented to search for a high-scoring subnetwork around SPP1. The procedure starts from SPP1 and proceeds by moving along the edges of the network to maximize $Z_S$. Moves are accepted according to the Metropolis criteria (41). Briefly, at every time step one neighboring node of the current subnetwork is picked with probability proportional to its $f_{es}$ score. If the $Z_S$ score change ($\Delta Z_S$) before and after the addition of the gene into the current subnetwork is positive, the addition of the gene is accepted. Otherwise, the addition is accepted with probability $P = \exp(\Delta Z_S/T_i)$, where $T_i$ is the current temperature for simulated annealing.

**Tissue collection and cell preparation.** Pathological oral tissues obtained from surgical resection and normal oral tissues obtained from plastic surgery were collected at West China Hospital of Stomatology, Sichuang University. The protocol was reviewed by the Institutional Ethics Committee of West China Hospital of Stomatology, and informed consent was obtained from each patient. The specimens were divided into several groups for different analysis described at the following sections. Group 1 (TG1) included 12 OSCC tissues and 12 normal oral tissues. Group 2 (TG2) comprised 127 OSCC tissues and 20 normal oral tissues. Group 3 (TG3) comprised 5 OSCC tissues and 5 normal oral tissues.
To obtain CAFs and NFs, the tissues were isolated, cultured, and purified using protocols described in Supplementary Materials. CAFs and NFs were examined using anti-pan cytokeratin (ZSGB-BIO; 1:100), anti-vimentin (Abcam; 1:200), and anti-α-SMA (R&D Systems; 1:200) antibodies. Two groups of fibroblasts were prepared to study SPP1 and TβRIII. Group 1 (CG1) included 12 CAFs and 12 NFs derived from TG1. Group 2 (CG2) contained 5 CAFs and 5 NFs derived from TG3. Three OSCC cell lines were prepared. Tca8113 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. SCC-25 cells were purchased from ATCC HSC-2 cells were from Tohoku University, Japan. HaCaT cells, human normal keratinocytes, were obtained from Cell Lines Service, Germany.

**Immunohistochemistry.** Anti-SPP1 (Abcam; 1:300) and anti-TβRIII (Santa Cruz Biotechnology; 1:200) antibodies were used to immunostain human OSCC and tumor-associated stromal sections derived from TG2 and TG3, respectively, according to the instruction of the Histostain-SP kit (Zymed). Immunostaining was scored using Kreisberg et al.’s scoring method (Supplementary Materials).

**DNA microarray analysis.** Agilent 44K Whole Genome Oligo Microarrays were used to measure the gene expression profiles of CG1. Sample preparation and hybrid reactions were performed according to the manufacturer’s instructions. RNA samples from CAFs and NFs were pooled respectively before hybridization. The chips were scanned and visualized using an Agilent DNA microarray scanner. Probe set intensities were measured using Agilent Feature Extraction software. Raw data were normalized by the Agilent GeneSpring GX software using the Agilent FE one-color scenario (median normalization). The data were deposited in the NCBI GEO.
database with accession number GSE35356.

**Real-time quantitative PCR and western blot analysis.** CG1, CG2, Tca8113, SCC-25, HSC-2, and HaCaT cells were analyzed by real-time quantitative PCR and western blot using standard protocols. SPP1 was amplified using 5’-CAGTTGTCCCCACAGTAGACAC-3’ and 5’-GTGATGTCCTCGTCTGTAGCATC-3’. TβRIII was amplified using 5’-TACAGAGAGAGGTCCACT-3’ and 5’-GTCTTCAGATGCCACACCAG-3’. SPP1 and TβRIII proteins were probed with anti-SPP1 (Abcam; 1:1000) and anti-TβRIII (Santa Cruz Biotechnology; 1:400) antibodies, respectively, which were further labeled with horseradish peroxidase-conjugated secondary antibodies (SPP: 1:5000; TβRIII: 1:20,000; both from ZSGB-BIO).

**Cell transfection and related assays.** CAFs in CG2, HSC-2, and Tca8113 cells were infected with lentivirus expressing TβRIII (PLenO-TβRIII-DCE-GFP) to generate CAF–TβRIII, HSC-2–TβRIII, and Tca8113–TβRIII cells, respectively. The same cells infected with retrovirus carrying an empty vector (PLenO-DCE-GFP) served as controls and are denoted as CAF–GFP, HSC-2–GFP, and Tca8113–GFP, respectively. Growth and viability of the transfected cells were assessed using Cell Counting Kit-8 (CCK-8; Dojindo). A colony-formation assay was performed to examine the long-time survival of the transfected OSCC cells after 10–15 days of growth. Apoptosis of transfected HSC-2 and Tca8113 cells was measured using the In Situ Cell Death Detection Kit-POD TUNEL system (Roche). Immunofluorescence analysis of α-SMA and immunohistochemistry of SDF-1 in fibroblasts were performed following anti-α-SMA (R&D Systems; 1:200) and anti-SDF-1 (Santa Cruz
Biotechnology; 1:100) antibody labeling. The ultrastructure of CAFs and CAF–TβRIII was compared using a transmission electron microscope.

**Subcutaneous tumorigenicity assays.** Thirty 12- to 14-week-old female BALBc \textit{nu/nu} nude mice were purchased from the experimental animal center at Sichuan University and were maintained under pathogen-free conditions with autoclaved food and distilled water. The mice were divided into six groups, each of which was coinjected subcutaneously on the right flank with different types of HSC-2 cells and fibroblasts (HSC-2+NF, HSC-2+CAF, HSC-2–TβRIII+CAF, HSC-2+CAF–TβRIII, HSC-2–TβRIII+CAF–TβRIII, and HSC-2–GFP+CAF–GFP). Tumor volumes were measured every 4 days one week after injection. All mice were sacrificed after the fifth measurement. Upon autopsy, the tumors were excised and weighed. Human specific anti-vimentin was used to detect CAFs and NFs in nude mice followed by immunohistochemical analyses with anti-Ki67 (Abcam; 1:600), anti-CD34 (Abcam; 1:25), and anti-E-cadherin (Abcam; 1:250) antibodies.

**Statistical analysis.** The Mann–Whitney test and Kruskal–Wallis test were used to evaluate differences between sample groups when appropriate.

**Supplementary materials.** Supplementary methods, tables and figures are available.

**RESULTS**

**Common Target Perturbation (CTP) Guided Epithelial–Mesenchymal Common**
Target (EMCT) Discovery Strategy

CTP concept hypothesizes that, in the development of cancer, epithelium and stroma share some important factors, and simultaneous attack on these common factors in both the epithelium and its adjacent stroma may augment the destruction of tumors. The key in the CTP-guided cancer therapy is the identification of effective common targets. If a gene exhibits significant expression changes in both the cancerous epithelium and its associated stroma compared to their normal counterparts, it would be important for cancer development, thus likely to be an EMCT. In addition, proteins rarely act in isolation. The biological functions of the proteins are implemented by interacting with other proteins, therefore each gene has its own topological characters. Combining both features, we defined an EMCT discovery strategy to screen highly potential EMCTs by evaluating gene expression behavior and topological significance in the context of the human protein–protein interaction (PPI) network.

This EMCT identification strategy is also developed to combine a series of experiments and computations into progressive organic cycles. In each cycle, computation-guided experiments and experiment-corrected computations will be performed to generate multiple feedbacks. Particularly, this feedback strategy allows different predictions to be proposed in each cycle, thus providing a flexible adjustment of candidates for experiments. Here, we implemented a prototypic, two-cycle procedure (Fig. 1). The first cycle identifies candidates important for cancer development with the expected expression behavior of EMCTs. This prediction further serves as seeds for the second round after verifying the expression of a seed. The second cycle searches for highly potential EMCT(s) that is closely interconnected around the seed. The derived computational scoring, weighted with database
knowledge, will finally determine the candidate as EMCT. Top ranked EMCT(s) will then be implemented into experimental verification and the final effective EMCT(s) will be confirmed.

**Prediction of SPP1 as an EMCT Seed**

A qualified seed gene should be heavily involved in cancer development. For example, genes that are dysregulated in many types of cancers will have a high likelihood of being involved in carcinogenesis. We adopted a two-step procedure to predict seeds. First, we analyzed the publicly available expression data on tumors to identify potential oral cancer-related genes that are over- or under-expressed in oral cancer; Second, considering that few data on oral stroma are available, we profiled the gene expression in oral CAFs and normal fibroblasts (NFs), and kept only those oral cancer-related genes that were differentially expressed in CAFs with respect to NFs as potential seeds.

To achieve a high success rate of prediction, we started from a list of reported 187 cancer signature genes, which were dysregulated in 20 types of cancers (17). We first ranked these genes by quantifying their importance in the context of the human PPI network. We defined a centrality index $N(D,s)$ for each signature gene $s$, which equals to the number of other signature genes located in its neighborhood within $D$-step distance. The assumption is that a signature gene might be more important if it has more and closer connections to other signature genes. Because of the small-world nature of the human PPI network (18), we only considered $N(D,s)$ for $D = 2$, i.e., a neighborhood two steps away from the central vertex. In our analysis, only those signature genes with $N(2,s)$ values $\geq 7$ were taken to be topologically important, which
generated a list of 24 genes (S24) (Supplementary Fig.1). Because oral cancer was not included in the selection of the original 187 genes, we further refined seed genes that show the common trend of expression changes in oral cancer as in other cancer types. We analyzed three public oral cancer data sets and kept 16 genes (S16) (Supplementary Fig.1).

To identify important genes functioning in the stroma during carcinogenesis, we collected 12 OSCC tissues and 12 normal oral tissues (TG1), from which oral CAFs and NFs (CG1) were obtained. We pooled the CAFs and NFs respectively, and measured their gene expression using microarrays. Based on our stroma expression data, seven genes (S7 out of the S16 set) differentially expressed in CAFs with respect to NFs were identified as seed predictions (Supplementary Fig.1). Two (SPP1, FAM107A) were consistently changed in the epithelium and stroma (i.e. with the same direction of change), and five (AURKA, PRC1, CEP55, FEN1, DNMT1) were inversely changed (i.e. with opposite direction of change). This S7 set represents highly potential EMCTs, which showed significant expression changes in the cancerous epithelium and its adjacent stroma, and were important in the sense of topological centrality. Lastly, with the consideration of further treatment on the epithelium and stroma simultaneously by the same agent, we selected SPP1 for further validation, which showed changes in the same direction in both cell types.

Experimental examination confirmed the expected upregulation of SPP1 mRNA and protein in both OSCC cells and CAFs compared with their normal counterparts (Fig. 2a). To verify the elevation of SPP1 level in clinical samples and its association with carcinogenesis, we examined the expression of SPP1 in 20 normal oral tissues and
127 OSCC specimens at different clinicopathological stages (TG2 in Methods) (Fig. 2b). In normal oral tissues, the level of SPP1 was nearly undetectable in stromal cells but increased in the epithelium with the progression of hyperplasia. By contrast, in about 87% of the OSCC samples, a strong SPP1 signal was detected in both the tumor and stromal cells. Correlation analysis indicated that the increase in SPP1 level was significantly associated with poor differentiation, large tumor volume, high clinical stage, lymph nodal metastasis, and recurrence, but not with sex, age, or tumor location (Supplementary Table 1).

**Seed Neighborhood Analysis Identifies **$\beta$**RIII as an EMCT**

After verifying the expression behavior of SPP1 and its close association with oral carcinogenesis, we proceeded to the second cycle of EMCT discovery to search for potential EMCT(s) that is closely interconnected around SPP1.

We introduced a score $f_{es}$ to quantify the extent of gene expression changes in the cancerous epithelium and stroma with respect to their normal counterparts. For a subnetwork $S$ of $k$ genes, we produced an aggregate score $f_S$ defined as the mean $f_{es}$ of all genes in the subnetwork. The final subnet score $Z_S$ is obtained by normalizing $f_S$ against the null distribution generated based on randomly sampled gene sets of size $k$. Based on the above scoring scheme, a Monte Carlo approach was implemented to search for a high-scoring subnetwork around SPP1 (Methods). The procedure starts from SPP1 and proceeds by moving along the edges of the network to maximize $Z_S$. Moves are accepted according to the Metropolis criteria. Because of its random nature, the procedure may give different results for different runs. To obtain a consensus subnetwork, we repeated the procedure 1,000 times and recorded the
number of times each gene was included in the results. We kept the top 1% high-frequency genes and obtained a connected subnetwork comprising 43 proteins and 80 PPIs (SPP1Net; Fig. 2c). Functional annotation indicated that SPP1Net is involved in cell adhesion, migration and blood vessel development (Supplementary Table 2). Members of several signaling pathways, including the TGF-β, MAPK, and ErbB signaling pathways are significantly enriched in SPP1Net.

The enrichment of members of the TGF-β pathway ranked first among all the examined signaling pathways, indicating the importance of the TGF-β pathway for epithelial–mesenchymal interaction during oral cancer development (Supplementary Table 2). As a top-level participant in the TGF-β pathway, TGF-β receptor III (TβRIII) had the highest $f_{es}$ score (0.99) in the pathway. In our previous work, we found that TβRIII was consistently downregulated in oral tumors and their adjacent stroma (19). The TβRIII level was suggested to be important for cancer tumorigenicity, progression, and invasion (20, 21, 22). Although no studies of TβRIII in stromal and oral cancer have been reported, our analysis indicated TβRIII as the top-rated candidate for final EMCT experimental validation. Upon completion of this work, we got to know a recent study, which reported that TGFBRIII downregulation could result in an immunotolerant tumor microenvironment (23).

**Common Target Perturbation (CTP) of TβRIII Effectively Inhibited Tumor Growth**

We first evaluated the expression level of TβRIII and confirmed the loss of TβRIII expression in both oral cancer epithelium and its adjacent stroma (Fig. 2d, 2e). To further assess whether targeting TβRIII is effective for tumor growth inhibition, we examined the effect of TβRIII ULP in oral cancer epithelial cells. We established two
OSCC cell lines re-expressing TβRIII (HSC-2–TβRIII and Tca8113–TβRIII) and two control cell lines expressing empty vector (HSC-2–GFP and Tca8113–GFP). Compared with the controls, the ULP groups exhibited a significantly lower cell survival rate (Fig. 3a) and colony formation efficiency (Fig. 3b), and a moderate level of apoptosis (Fig. 3c). These observations indicated that re-expression of TβRIII in human oral cancer cell lines decreased cancer cell proliferation.

To investigate the effect of TβRIII ULP in stromal cells, we established an oral CAF cell line re-expressing TβRIII (CAF–TβRIII) and a control cell line expressing the empty vector (CAF–GFP). Cell viability did not change significantly between the ULP and control groups (Fig. 3d). However, the ULP group showed down-regulation of CAF markers including α-SMA (Fig. 3e) and SDF-1 (Fig. 3f), suggesting potential structural alterations in CAF–TβRIII cells. Structural analysis showed that certain typical morphological structures, such as large spindle-like shape, intended nuclei, peripheral myofilaments and focal densities were present in the CAF and CAF–GFP cells but appeared absent in the CAF–TβRIII cells and NFs (Fig. 3g). These results indicated that some specific ultrastructures of TβRIII ULP in CAFs have changed, which are more close to those of NFs.

After perturbation of TβRIII expression in oral cancerous epithelial cells and CAFs, we continued to explore whether CTP-targeting TβRIII in epithelium and stroma simultaneously would be more effective than the ULPs in suppressing cancer growth. Six in vivo xenograft models were constructed to implement different combinations of human oral cancer cells and fibroblasts: HSC-2+CAF, HSC-2–GFP+CAF–GFP, HSC-2+NF, HSC-2+CAF–TβRIII, HSC-2–TβRIII+CAF, and
HSC-2–TβRIII+CAF–TβRIII. As shown in Fig. 4a, the tumor volume was greater in the HSC-2+CAF group (2827.0 ± 1195.7 mm$^3$) than in the HSC-2+NF group (1064.3 ± 260.4 mm$^3$). Tumors derived from the ULP groups, HSC-2–TβRIII+CAF and HSC-2+CAF–TβRIII, had smaller volumes (656.9 ± 234.1 mm$^3$ and 852.8 ± 361.5 mm$^3$, respectively) compared with those from the unperturbed groups, HSC-2+CAF and HSC-2+NF. Tumors from the CTP group HSC-2–TβRIII+CAF–TβRIII had the smallest volume (541 ± 171 mm$^3$). These findings demonstrated that the CTP administration of TβRIII to both tumor and stroma cells significantly suppressed tumor growth in vivo and was more effective than the ULP approach.

To understand the biological changes in the process of TβRIII CTP, we performed further investigations. Expression of the proliferation marker Ki-67 was reduced markedly in TβRIII-perturbed groups but did not show significant difference between the ULP and the CTP groups (Fig. 4b). The average microvascular density in TβRIII-perturbed groups was significantly lower than in the unperturbed groups. Importantly, the CTP group had the lowest density, suggesting stronger suppression of tumor angiogenesis by TβRIII CTP (Fig. 4c). Pathological examination of the primary tumors demonstrated that TβRIII CTP decreased tumor metastasis (Fig. 4d). Examination of E-cadherin expression, which correlates negatively with the potential for tumor invasion and metastasis, showed much higher level of E-cadherin in the CTP group compared with other groups (Fig. 4e), indicating a substantial depression of invasiveness. In all, these data indicated that the superiority of TβRIII CTP over ULP in tumor suppression was caused by depression of tumor proliferation, angiogenesis, and metastasis.
DISCUSSION

In recent years, the complex tumor-microenvironment interactions have been recognized as important contributors to the robustness of cancer (24-26) and viable targets for therapeutic intervention (8, 27, 28). In light of the BLP combinatorial strategy (7-9), we proposed the CTP concept as a potential anticancer therapy, which has the advantage of perturbing the same target in the epithelium and its stroma simultaneously by the same agent. We developed a systems biology strategy that progressively cycles experiments and computations to discover EMCTs, the common ‘fragile key points’ for both the cancerous epithelium and its adjacent stroma. In the present work, a two-cycled EMCT discovery strategy was implemented to identify EMCTs in both the oral cancerous epithelium cells and their adjacent CAFs and two types of ‘omics’ data (expression profiling and PPIs) were used. As demonstrated, this multiple feedback strategy helps adjust experiment and computation schedule flexibly and lead to a greater success rate of target discovery. Although we only focused on TβRIII on the basis of a combination of different criteria among the predicted EMCTs, more genes can be identified. Other potential EMCTs could be validated with further work. For instance, FAM107A, identified in our cycle I analysis, is likely to be one potential target, since several recent reports have demonstrated the involvement of its interacting partner PPP2R2A in cancers (29, 30). Besides, the framework is flexible to integrate additional ‘omics’ datasets from different cell types. Our initial attempt to identify therapeutic targets by considering the tumor and its surrounding microenvironment simultaneously has shown promise on future cancer therapy.

The EMCT discovery strategy offers an important application of systems biology.
Biological systems are complex systems composed of a huge number of interacting components (31). Investigating biological systems from a systems perspective has important implications for understanding cancer and devising effective therapies (32). A crucial factor that underlies the efficacy of a therapeutic intervention is the robustness of the target system (24). This ability of resistance to perturbations is attained through various mechanisms. For example, functional redundancy could enable a tumor to sustain drug attack on a biochemical pathway by finding alternative routes to escape the blockage (33). This redundancy partly contributes to inefficiency of current therapeutic strategies. Modularity, another fundamental property of biological systems, provides an additional mechanism to localize perturbations to minimize the effects on the whole system (34). Although generally robust, biological systems can also exhibit fragility at certain key points, attacks on which are destructive for the systems (35). However, given the complexity of the human genome and the network that underlies the pathology, identifying the points of fragility is challenging. Our results demonstrates systems biology as a powerful tool to explore complex biological systems, which can help find the Achilles’ heel of the pathological systems and design effective therapeutic strategies.

In addition, this study identified TβRIII as strong target for cancer treatment. Our experiments indicated that TβRIII CTP is more effective than TβRIII ULPs in either cell type. On one hand, as shown in TβRIII ULP in oral cancerous epithelial cells, the viability of tumor was significantly decreased suggesting that TβRIII is a point of fragility for cancerous epithelial cells. On the other hand, TβRIII ULP in CAFs changed the phenotype of CAFs to that of NFs. It indicates that TβRIII offers vulnerability for CAFs and its perturbation promoted the transformation from the
pathological state of CAFs to the normal state of NFs. This phenotype transformation impaired the support of CAFs to the tumor, and leaded to the observed strong depression of angiogenesis and metastasis in TβRIII CTP. Therefore, if attacking a correct target, the CTP concept could achieve a superior efficacy over the ULP approach by destroying the tumor in conjunction with the support from its surrounding microenvironment. In addition, when altering the system-wide behavior by targeting one single element is not achievable, an improved CTP strategy could be applied by performing multi-target perturbation which can aim at different scales, such as signaling and metabolism, to allow more effective treatment.

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AUTHOR CONTRIBUTIONS

H.Z. and Z.L. initiated the hypothesis and designed the research. W.M. and Y.W., L.G., C.L., Y.L. and Y.G. carried out all the experiments. H.Z., Q.G and L. W. supervised the experiments. X.L., Y.L., S.C. and X.K contributed reagents, materials and analysis tools. X.H. and Z.L. performed the computations. Z.L. and H.Z. wrote the manuscript with the help of W.M., Y.W. and X.H.
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**FIGURE LEGENDS**

**Figure 1** The strategy of epithelial–mesenchymal common target (EMCT) discovery.

The two-cycle procedure implemented in this study is shown. The first cycle identifies candidates serving as seeds and the second cycle searches for highly potential
EMCT(s) that is closely interconnected around the seed. CAF, carcinoma-associated fibroblast; NF, normal fibroblast; PPI, protein-protein interaction.

**Figure 2** Verification of the expression behavior of SPP1 and TβRIII and identification of the PPI subnetwork around SPP1. (a) SPP1 was upregulated at the mRNA (left) and protein (right) levels in both OSCC cells and CAFs (CG1) compared with normal epithelial cells and NFs (CG1). Error bars are means ± s.e.m. (n = 3 for epithelial cells, n = 12 for fibroblasts); # P = 0.06 , *** P < 0.001. (b) Immunohistochemical analysis showed that the SPP1 levels in the epithelium and stroma increased with the progression of clinicopathological stage. In normal epithelium, SPP1 was nearly undetectable (1). SPP1 was dispersed in hyperplastic epithelium but rarely detected in stromal cells (2). For highly differentiated OSCC, SPP1 was detected in both tumor cells and stromal cells (3). The positive staining was extended in infiltrated OSCC, with moderate to strong signal in the tumor invasion front (4). SPP1 was expressed strongly in both the tumor and its adjacent stroma for poorly differentiated OSCC (5) and OSCC with lymph node metastasis and recurrence within 3 years after surgery (6). (c) The identified PPI subnetwork around SPP1 (SPP1Net). (d) Immunohistochemical analysis confirmed downregulation of TβRIII in oral cancerous epithelium and stroma compared with their normal counterparts. (e) Compared with normal epithelial cells and NFs (CG2), both OSCC cells and CAFs (CG2) exhibited less TβRIII at the mRNA (left) and protein (right) levels. Error bars are means ± s.e.m. (n = 3 for epithelial cells, n = 5 for fibroblasts); * P < 0.05.

**Figure 3** Unilateral perturbation of TβRIII in OSCC cells and CAFs. (a) Examination
of cell proliferation by CCK-8 assay showed that proliferation of Tca8113–TβRIII and HSC-2–TβRIII cells was significantly decreased. (b) A colony-formation assay examining long-term OSCC cell survival showed a marked decrease in the number and size of cancer cell colonies for Tca8113–TβRIII and HSC-2–TβRIII cells. Error bars are means ± s.e.m. (n = 3); * P < 0.05. (c) Apoptosis analysis by TUNEL assay indicated that re-expression of TβRIII induced apoptosis in Tca8113–TβRIII and HSC-2–TβRIII cells. Red arrows indicate apoptotic bodies. (d) Cell proliferation did not differ between CAF–TβRIII cells and control groups (CCK-8 assay). (e) α-SMA and (f) SDF-1 were downregulated in CAF–TβRIII cells. (g) Structural analysis by transmission electron microscopy showed that the high expression level of TβRIII in CAF–TβRIII cells was associated with the disappearance of some characteristic features present in CAFs and CAF–GFP cells, for instance, peripheral myofilaments (red arrows) and focal densities.

**Figure 4** Effective inhibition of tumor growth through the CTP concept targeting TβRIII in OSCC cells and CAFs simultaneously. (a) Tumor volumes and weights decreased in TβRIII-perturbed cells. The CTP group had the smallest tumor volume and lowest weight. Error bars are means ± s.e.m. (n = 5); *** P < 0.001. The CTP group had a significant lower tumor weight compared with the ULP group in epithelial cells (P=0.01) and fibroblasts (P=0.004). (b) Intratumoral Ki-67 expression was significantly decreased in TβRIII-perturbed cells. (c) TβRIII perturbation significantly inhibited the formation of tumor-associated microvasculature. Blood vessels were visualized by immunohistochemistry with CD34. The average microvascular density was significantly lower in the CTP group than in the non-CTP groups (P= 0.004 for ULP in epithelial cells, P=0.004 for ULP in fibroblasts). Error bars are means ± s.e.m.
(n = 5); *** \( P < 0.001 \). **(d)** Re-expressing TβRIII decreased tumor metastasis. (1–3) Tumors from the HSC-2+CAF group exhibited increased invasion of the local tissue and surrounding muscle tissue. (4–5) Tumors from the HSC-2–TβRIII+CAF and HSC-2–TβRIII+CAF–TβRIII groups exhibited little to no invasion and instead maintained a distinct margin with the adjacent normal tissue. (6–9) Because of the short-term observation, no damage or tumor metastasis was observed in main organs (heart, liver, lung and kidney) in mice. **(e)** Intratumoral E-cadherin expression increased substantially in the CTP group.
Figure 1

Signature genes dysregulated in twenty types of cancers excluding oral cancer (187 genes)

- Identification of genes with topological significance
  - 24 genes (524)
- Downloaded gene expression profiles of oral cancer
- Identification of genes with consistent direction of expression changes in oral cancer as in other types of cancers
  - 16 genes (516)
- Identification of genes with significant expression changes in CAFs with respect to NFs
  - 7 genes (57)
- Identification of genes with consistent direction of expression changes in oral cancer and CAFs with respect to their normal counterparts
  - 2 genes (52)

Human protein-protein interactions

Functional annotation

Prior knowledge

Evaluation of predictions

Expression verification of the selected candidate TIRBI

In vivo unilateral and bilateral perturbation experiments of TIRBI

Confirmation of TIRBI as an effective EMCT
Figure 3
Figure 4

(a) Graph showing tumor volume (left) and tumor weight (right) over days after injection. 

(b) Microscopic images of different samples, showing variations in tissue structure.

(c) Bar graph indicating the microscopic density of different samples, with significant differences marked.

(d) Additional microscopic images with numerical labels 1 to 4.

(e) Close-up images of specific areas from different samples.
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